# ASPECTS OF
# EUKARYOTIC DNA REPLICATION, REPAIR AND RECOMBINATION

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Xin Li

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ASPECTS OF

EUKARYOTIC DNA REPLICATION, REPAIR AND RECOMBINATION

Xin Li, Ph. D.

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The mechanism that replicates, maintains, and sometimes alters the DNA is most fundamental and important for life. Three processes (DNA Replication, Repair, and Recombination) that are involved in this mechanism are closely related and well conserved in evolution. In my Ph.D. studies, I have used the cross model-organism approach to investigate the molecular mechanisms of DNA replication stress induced cancer as well as meiosis disruption caused infertility.

*Mcm4*$^{Chaos3}$, which encodes a mutant subunit of the hexameric MCM helicase, was previously reported to cause genetic instability (GIN) in mice and predispose homozygous female mice to mammary adenocarcinomas. My results show that homozygous diploid yeast carrying the equivalent mutation are defective in replicating long terminal repeat (LTR) elements. The replication stress at these interspersed repeat sequences coupled with error prone repair is the source of GIN, which is the driving force for the acquisition of an accelerated proliferation (AP) phenotype with aneuploidy as byproducts. I showed that mutations unrelated to aneuploidy are the cause of the AP phenotype. Moreover, the fragility of LTR regions is dependent on ploidy. The LTRs are not vulnerable to replication stress in haploid yeast and *mcm4*$^{Chaos3}$ haploids use other repair pathways without generating GIN. Therefore, the dichotomy of consequences of DNA replication stress and repair pathway choice stems from cell-type specific regulation of fragile sites.

In mammalian meiosis, homologous chromosome synapsis is coupled with recombination. As in most eukaryotes, mammalian meiocytes have checkpoints that monitor the fidelity of these processes. I reported that the mouse ortholog (*Trip13*) of pachytene checkpoint 2 (*PCH2*), an essential component of the synapsis checkpoint in *S. cerevisiae* and *C. elegans*, is required after strand invasion for completing a subset of recombination events, but possibly not those destined to be crossovers (Li and Schimenti 2007). TRIP13-deficient mice exhibit spermatocyte death in pachynema and loss of oocytes around birth. The chromosomes of mutant spermatocytes synapse fully, yet retain several markers of recombination intermediates. This is the first model to separate recombination defects from asynapsis in mammalian meiosis. Surprisingly, we found no evidence for checkpoint function, suggesting different pachytene checkpoint mechanisms may be involved in different species (Li et al. 2008).

## BIOGRAPHICAL SKETCH

Xin Li was born in Tianjin, China in 1982. His interests in life science were revealed at an early age. As a high school student, he was sent to Nankai University (60 credits) & Tianjin Normal University (67 credits) for one-on-one special training in biology during 1998-1999. He got the Gold Medal in the 8th National Biology Olympiad in 1999, which allowed him to study Biological Science and Biotechnology at Tsinghua University with an entrance exam waiver from 2000 to 2004. During his undergraduate studies, he built up a solid background in mathematic, physics and chemistry. He was a pioneer in student-independent research, having sought and received his own grants, equipment, research students, and laboratory. As the President of the Students' Science & Technology Association of Biology Department, he organized a series of events to promote an academic atmosphere. With the highest awards for research and academic performance at his college, he was recruited by Cornell University to pursue his Ph.D. in the field of Biochemistry, Molecular, and Cell Biology field in 2004.

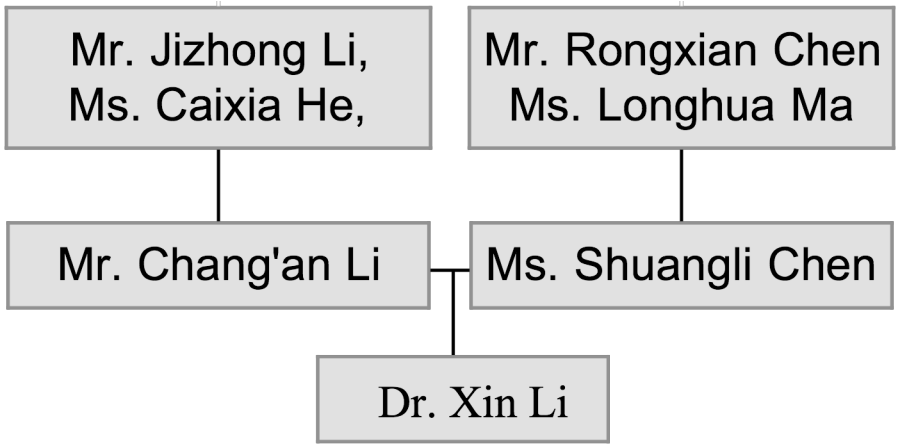
After he rotated in the laboratories of Robert Weiss, Bik Tye, Jeff Robert, and John Schimenti, Xin Li decided to work as a joint student under both Bik Tye and John Schimenti, with half of the pay from each lab. The initial idea was to use both yeast and mice as model systems to study the cancer induced by a novel allele of MCM4. At that time, the phenotype of the allele he worked on, $mcm4^{Chaos3}$, had been extensively studied in yeast, and no genetic instability was found. After months of research in the Tye Lab, he found that this allele has a special defect in diplophase, causing a G2/M delay. At that time, no one believed him, but he persisted in his belief. One thing led to another, and half of his Ph.D. thesis originates from that initial piece of discovery. In the Schimenti lab, besides the MCM project, he was given another project on mouse *Trip13*, an ortholog of yeast pachytene checkpoint protein, right

after he joined the lab. Before him, others had carried out the project, but little progress was made. Fortunately, the *trip13* mutant mice yielded very interesting and unexpected results. A novel phenotype combined with his hard work allowed him to publish the results less than two years in Plos Genetics, far ahead of his competitor from Scott Keeney Lab. The paper then won the LPS Best Paper Award in 2007. After the paper on *trip13*, he found it hard to continue to pursue this project. He spent almost a year of exploration. Based on the results of having characterized a double mutant of *trip13* with another meiotic protein, he decided to focus on this other protein, Sycp3. Before his thesis defense, he additionally developed a preliminary story concerning this protein, which was not included in his thesis.

During Xin Li's Ph.D. study, he has enjoyed an experiential connection with the principles of life and he has decided to stay in the field of academia to answer fundamental biology questions in the hope that they will eventually benefit others. During his time working on the Ph.D., Xin Li has built respectful relationship with his mentors, interacted with other researchers inside and outside of Cornell University, and has constantly updated himself by taking new courses every semester. Due to the popularity of the name "Xin Li", he added "Chenglin" as his middle name for his publication.

At Cornell University, Xin Li explored himself beyond research by taking courses in outdoor activities, business, dancing, wine, and piano. He worked as a volunteer in the Cornell animal hospital and got accepted to the DVM program of Cornell Vet School in 2006. As he arrived at America, he further discovered the beauty of traditional Chinese culture, including Confucianism, Taoism and Buddhism, which has led him to pursue the true nature of life and universe ever since.

I dedicate my thesis to my family and friends who give me love and support.



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# CHAPTER 1

# INTRODUCTION

## *Genetic instability and DNA replication*

Genetic instability (GIN), a hallmark of cancers (Loeb 2001; Rajagopalan et al. 2003), refers to a series of observed spontaneous genetic changes occurring at an accelerated rate in cell populations derived from the same ancestral precursor (Raptis and Bapat 2006). Two types of GIN have been characterized (Lengauer et al. 1998). The first arises from a defect in mismatch repair which results in successive widespread microsatellite instability (MIN) (Perucho 1996). The second is chromosomal instability (CIN), the consequence of which is an increase in the rate of loss of heterozygosity (LOH) and/or aneuploidy (Michor et al. 2005). There are two ways in which cells can become aneuploid: whole chromosome gain or loss, which originates from errors in cell division, and rearrangements in chromosome structure, arising from breaks in DNA (Pellman 2007). MIN and CIN mechanisms are generally found to be mutually exclusive and to produce different phenotypes (Lengauer et al. 1997; Raghavan and Lieber 2006). The role of MIN in human cancer is well established (Strate and Syngal 2005). However, the contribution of CIN is less certain (Pihan and Doxsey 2003).

The roles of GIN in tumorigenesis are still under debate (Sieber et al. 2003). It is believed that GIN accounts for the numerous mutations in tumors (Renan 1993). GIN is a general term to describe the overall process that increases the rate of mutation, thus enabling cell(s) to develop new and aggressive phenotypes and to adapt to the changing selection pressures (Bayani et al. 2007). Supporting the notion that GIN is the driving force for tumorigenesis, it was found that GIN is an early event in the progression of at least some cancers (Bartkova et al. 2005b), and that DNA repair,

chromosomal segregation, and checkpoint defects can predispose animals and human to cancer (Hoeijmakers 2001). However, studies in model organisms and cell cultures indicate that GIN is directly associated with growth/developmental defects, cell death, or senescence (Burhans and Weinberger 2007). Given the deleterious nature of GIN, it has also been argued that GIN is a consequence rather than the initial driving force of tumorigenesis (Tomlinson and Bodmer 1999).

Exploring the origin of GIN is essential to understand the early events in tumor development. DNA replication stress has been proposed to be a source of GIN (Schar 2001). However, because of the multiple functions of replication proteins and the lethality of most DNA replication mutants, little is known about whether and how DNA replication defects initiate GIN, and whether such defects underlie GIN observed in many cancers. The *Mcm4*$^{Chaos3}$ (F341I) mutation, recently discovered in mice, provides a tool to address these questions.

*Mcm4*$^{Chaos3}$ was identified in a forward ENU mutagenesis screen for mice exhibiting chromosome instability (Shima et al. 2003). The screen used flow cytometry to detect micronuclei in erythrocytes (Dertinger et al. 1996). These micronuclei are the products of chromosome breakage and aneuploidy events (Nusse et al. 1996). One of the mutations identified, *Chaos3* (chromosome aberrations occurring spontaneously 3), turned out to be an allele of *Mcm4* (Minichromosome maintenance deficient 4). Female mice homozygous for *Mcm4*$^{Chaos3}$ are highly prone to mammary tumors with a mean latency of 12 months (Shima et al. 2007). This ubiquitous defect in the MCM helicase seems to target only mammary tissues in female mice for cancer development in a particular strain. Thus, unlike other transgenic mouse models, the homozygous *Mcm4*$^{Chaos3}$ mice provide an excellent model for studying mammary tumor development in mammals caused by a single

amino acid change of MCM4.

*MCM* genes were first identified in a screen for yeast mutants unable to stably maintain minichromosomes containing a single replication origin (Maine et al. 1984). The Mcm2-7 proteins, which together form a heterohexameric ring, are believed to function as a DNA helicase in both DNA replication initiation and elongation (Maine et al. 1984; Tye 1999; Forsburg 2004; Moyer et al. 2006). The six structurally-related MCM proteins, all of which are essential for viability, are conserved throughout eukaryotes (Tye 1999; Forsburg 2004). Recent studies suggest that the MCMs may also play a role in DNA repair (Shukla et al. 2005) and DNA damage response (Malumbres and Barbacid 2001; Cortez et al. 2004; Bailis et al. 2008). Since MCM proteins are expressed at high levels in proliferating cells but not at all in quiescent cells, they have emerged as potential diagnostic pre-cancer markers (Gonzalez et al. 2005). The studies of $Mcm4^{chaos3}$ provide the first evidence that defects in MCM proteins themselves cause cancer. However, due to the multiple functions of MCM proteins, it is hard to dissect the *in vivo* initial consequence of altering a specific amino acid (F341I) in MCM4 that ultimately predisposes mice to cancer.

A more intriguing question is why and how a defect in the MCM replicative helicase targets mammary tissue but no other tissues for cancer development in mice in C3H background. A DNA replication defect does not always lead to GIN. In $Mcm4^{Chaos3/Chaos3}$ mice, the mammary gland of female mice and progenitors of erythrocytes are the only tissues known to be affected (Shima et al. 2007); otherwise the mice are grossly normal. What tips the balance causing $Mcm4^{Chaos3}$ induced defects to favor GIN in some tissues, whereas in other tissues toward normality?

Basic molecular mechanisms that underlie DNA replication, DNA damage checkpoint and DNA repair pathways are well conserved in evolution (Nyberg et al.

2002). This conservation allows the use of a diverse set of model organisms to address basic questions about human cancer. The *Mcm4*$^{Chaos3}$ allele contains a single point mutation in a region of Mcm4 that is highly conserved from *Archaea* to humans and that specifies the interface of adjacent subunits (Fletcher et al. 2003). This strong conservation allows us to introduce the corresponding *mcm4* mutation into yeast *MCM4* and use yeast as a model organism to investigate the complex molecular mechanism of *Mcm4*$^{Chaos3}$-induced cancer in mice.

The properties of the *Mcm4*$^{Chaos3}$ mouse mutant are recapitulated in the diploid yeast carrying the equivalent *mcm4*$^{Chaos3}$ mutation. The *mcm4*$^{Chaos3}$ diploid yeast exhibits G2/M delay with severe GIN, but the haploid *mcm4*$^{Chaos3}$ mutant is grossly normal without obvious GIN (Chapter 2 and 3). Our study shows that only in diploid yeast, the LTR (long terminal repeat) regions are vulnerable to replication stress induced by *mcm4*$^{Chaos3}$. Therefore, using haploid mutant as a negative control, I am able to draw the conclusion that DNA replication defect at the interspersed repeat sequences, LTRs, coupled with error prone repair, is the cause of GIN in *mcm4*$^{Chaos3}$ mutant, an explanation that could be extended to the mouse *Mcm4*$^{Chaos3}$ mutants. The cell type specificity of GIN induced by replication stress stems from the regulation of replication at the interspersed repeat sequences.

***Fragile site and LTR regions***

Previously, it was believed that inhibition of replication affects the whole genome equally. However, the identification of fragile sites has challenged this idea (Magenis et al. 1970). Fragile sites are regions of genome that are particularly prone to breaks following partial inhibition of DNA synthesis. Based on the population frequency and pattern of inheritance, fragile sites can be divided into rare fragile sites (RFSs) and common fragile sites (CFSs). In contrast to RFSs that are only seen in less than 5% of the human population, CFSs are seen in all individuals. While the

increased breakage of RFSs is mostly due to expansion of nucleotide repeats, the reason for the fragility of CSFs is still unclear.

The CFSs, encompassing very large genomic regions, are frequently rearranged or deleted in tumor cells (Durkin and Glover 2007; Freudenreich 2007; Smith et al. 2007). While there are some weak links between the genes at the CFSs, for example FHIT and WWOX, and their tumorigenic functions, the significance of this association is still unclear. Although CFSs are not involved in the most commonly recurrent translocations in cancer and leukemia, the fact that precancerous proliferating cells frequently express CFSs makes these sites to be the most likely earliest chromosomal changes associated with cancer (Bartkova et al. 2005a; Gorgoulis et al. 2005). Moreover, CFSs are conserved throughout mammalian evolution (Durkin and Glover 2007), suggesting a yet-to-be identified conserved purpose of these regions that might be related to its potential role in tumorigenesis.

Given the sensitivity of CFSs to replication elongation stress, they may represent vulnerable regions of the genome where replication forks are more likely to stall or collapse. Their fragility are likely related to secondary structure formation, interference with nucleosome assembly and late replication (Durkin and Glover 2007; Lukusa and Fryns 2008). Recent discoveries that Ty elements and LTRs are hotspots for translocation in yeast under replication stress (Dunham et al. 2002; Lemoine et al. 2005; Argueso et al. 2008; Lemoine et al. 2008; Li et al. Submitted) suggest that both yeast and mammalian genomes exhibit replication-stress-sensitive loci. The Ty and LTR elements may be functionally analogous to mammalian CFS and provide a potential model to understand the mechanisms of chromosome fragility.

Under reduced levels of DNA polymerase, it was found two head-to-head Ty elements are preference sites for DSBs and rearrangements (Lemoine et al. 2005;

Lemoine et al. 2008). Another yeast fragile site contains a region with two tRNA genes and head to head LTRs (Admire et al. 2006). It was proposed that under replication stress, the decoupling of DNA helicase and polymerase exposes ssDNA, which may form secondary structure, such as hairpins, serving as barriers for polymerase that induce breaks at CFSs. In this study, I found that solo LTR regions are vulnerable to replication fork stalling and are hotspots for recombination in *mcm4*$^{Chaos3}$ mutant, which have challenged this model (Chapter 1). First, LTR elements alone, rather than the whole Ty elements serves as fragile sites. Second, most of the fragile sites I identified do not have the head to head orientation, so a hairpin structure is unlikely to be a barrier. Third, the source of replication stress in our study is a defective helicase bearing the *mcm4*$^{Chaos3}$ mutation, which counters the hypothesis of polymerase-helicase uncoupling as an initial event in CFS instability. In this scenario, DNA structure barriers would impede helicase, rather than polymerase progression. Moreover, I found that fragility of LTRs is dependent on ploidy, suggesting that the epigenetic regulation of these regions, rather than secondary structure, determined the fragility.

Ty elements are LTR-retrotransposons that replicate through an RNA intermediate and are representative of a class of mobile genetic elements existing in all eukaryotes. In *S. cerevisiae*, there are about 300 Ty related elements each flanked by LTRs clustered in about 30 - 40 locations (Gabriel et al. 2006). In addition, there is an order of magnitude more solo LTR elements (Gabriel et al. 2006). Altogether, these repetitive sequences represent about 3% of the genome. Ty elements encode about 10% of the total mRNA in haploid *Saccharomyces cerevisiae*, and alter the expression of the adjacent genes (Servant et al. 2008). It is known that most Ty elements are cell-type regulated, and their transcription is repressed in diploid cells (Company and

Errede 1988; Fulton et al. 1988; Bilanchone et al. 1993; Morillon et al. 2000), which is partially if not completely regulated by *MAT* locus heterozygosity (Wilke et al. 1992).

A Ty element sometime recombines between LTRs deleting the whole Ty element and leaving a solo LTR as a "scar". The LTR regions contain a complex array of positively and negatively acting sequences. The Ty1 and Ty2 LTR contain an upstream activation site (UAS) and two TATA sites (Liao et al. 1987). In the Ty5 LTR, there are several pheromone responsive elements (PRE) that are responsible for the transcriptional repression of Ty5 (Ke et al. 1997). The Ty3 LTR contains a negative control region, PRE, and a1/α2 binding sites (Bilanchone et al. 1993). The function of LTR as replication barriers in diploid as demonstrated in our previous study probably depends on the interaction of the proteins bound at these sites, whose binding influences the local chromatin structure. Previous studies of retrotransposons have focused on the entire elements; here I showed that solo LTRs not only provide regions of portable homology for recombination, but also play an important role on the cell type specific response to replication stress.

Repetitive sequences and transposable elements are enriched in heterochromatin and undergo epigenetic regulations (Peng and Karpen 2008). Cosuppression is the high copy number-triggered silencing of dispersed homologous genes (Jorgensen 1995), which may evolve in eukaryotes to control molecular parasites such as viruses and transposons (Wolffe and Matzke 1999). Cosuppression is a fairly common process through mainly posttranscriptional gene silencing (RNAi) and transcriptional gene silencing. Such cosuppression also control Ty1 expression and the promoter of Ty1 is required for this negative feedback control (Jiang 2002). Recently, it was found that an anti-sense cryptic transcript encompassing the Ty1 LTR mediates the silencing of Ty1, through a RNA-dependent mechanism which is similar

to heterochromatin gene silencing (Berretta et al. 2008). MCM genes also seem to be involved in this cosuppression directly. It has been shown that *mcm5* mutants have upregulated expression of Ty proximal genes and defects in the establishment of compact chromatin domains near Ty elements (Dziak et al. 2003). This raises the interesting possibility that the Ty regions may not be silenced in *mcm4*$^{Chaos3}$ diploid mutants and the change in chromatin structure might be a potential mechanism for LTRs as fragile sites in diploid mutant.

Similar to Ty elements in yeast, Alu repeats, short interspersed elements, are the most abundant family of retroposons in human (11% of human genome). A subset of Alu repeats are still mobile and could cause genetic variability and heritable disorders (Schmid and Maraia 1992). A genome wide mapping of the CFSs caused by oncogene-induced replication stress showed that CFSs and regions with LOH were enriched with Alu repeats (Tsantoulis et al. 2008), suggesting that Alu elements may behave as fragile sites as LTR regions do. Interestingly, many Alu repeats contain a novel class of estrogen receptor (ER) binding elements with a high affinity for ER that work as estrogen receptor-dependent enhancers (Norris et al. 1995). The upregulation of the BRCA1 mRNA in human breast cells is mediated by this Alu-associated estrogen response element (ERE) in BRCA1 gene promoter (Tomilin 1999), and recombination of Alu repeats can result in *BRCA1* deletion. These results, together with our finding on the cell type regulation of fragile sites in yeast, raise the interesting possibility that the binding of the ER to the Alu sequences in mammary gland may cause stronger fragile sites than other tissues, making the mammary gland in the mutant more vulnerable to disruption of fork integrity.

***Repair pathway choice to maintain fork integrity***

Cells have developed multiple pathways to deal with a particular type of DNA damage. These pathways are distinct regarding repair efficiency and mutagenic potential and must be tightly controlled to preserve viability and genomic stability. Inappropriate repair have dire consequences including diseases. As I gain a better understanding of the mechanism that regulates repair pathway choice in different cell types, it is likely that basic mechanistic insights will eventually translate into clinical benefits. So far, most of our understanding of repair pathway regulation comes from the studies of double strand breaks (DSBs) (Shrivastav et al. 2008b).

Double strand breaks (DSBs) are repaired by two main pathways, nonhomologous end-joining (NHEJ) and homologous recombination (HR). Yeast mainly uses the HR pathway. In diploid yeast, the NHEJ is severely disabled through the repression of *NEJ1,* the key components of NHEJ, by transcription factor a1-α2 coded by *MAT*a/α (Frank-Vaillant and Marcand 2001). While human somatic cells use NHEJ as the main pathway to repair DSBs (Mao et al. 2008), embryonic stem cells display enhanced HR capacity. Because of this capacity for HR, embryonic stem cells are amenable to direct genetic manipulation. However, little is known about the cell type specific regulation for damage repair other than DSBs (Barbour and Xiao 2006; Shrivastav et al. 2008a).

A replication fork defect may originate from natural replication barriers, environmental insults, intrinsic errors of cellular metabolism, or DNA transactions. Prolonged stalling of DNA replication could result in DSBs. Cells have evolved alternative pathways to enable fork restarts away from the origin of replication. Some pathways operate through recombination mediated replisome reassembly and some operate directly at stalled forks (Barbour and Xiao 2003; Heller and Marians 2006).

Recombination mediated DNA replication is an ancient process adopted by both prokaryotes and eukaryotes. Spontaneous DNA breakage is a frequent, inevitable consequence of DNA replication that occurs at about 1% per cell division in *E.coli*, (Pennington and Rosenberg 2007). The recombination machinery is believed to evolve as a fundamental component of the basic replication machinery rather than an auxiliary adaptation for rare repair of certain DNA damage (Cavalier-Smith 2002b). The assembly of a replication fork by recombination-mediated processes requires many replication elongation proteins (Paques and Haber 1999).

*RAD6* dependent fork resumption pathway is unique to eukaryotic cells, suggesting that this pathway is a recent evolutionary innovation. Distinct from the recombination-mediated replication, this pathway operates through a bypass mechanism without reassembly of the fork. Rad6-Rad18 complex (Bailly et al. 1994) and the Mms2-Ubc13-Rad5 complex (Ulrich and Jentsch 2000) are the two E2 (ubiquitin-conjugating) -E3 (ubiquitin-ligase) enzyme complexes essential in this pathway. They bind the exposed single-stranded DNA, and mediate the ubiquitination of the stalled replication machinery. Proliferating cell nuclear antigen (PCNA) is one of the targets for this repair pathway, and different modifications affect the resistance to genotoxic insults on the forks (Hoege et al. 2002).

The PCNA poly-ubiquitination leads to “gap filling” repair, using the newly synthesized sister chromatin as a template to bypass genotoxic insults presumably through a template switching mechanism. A damage tolerant pathway through PCNA mono-ubiquitination recruits an error prone DNA polymerase, which enables the fork to bypass the damage (Lee and Myung 2008). Small ubiquitin-like modifier (SUMO) modifies PCNA to recruit the SRS2 helicase, which prevents recombinational repair from inappropriately resolved stalled replication forks (Papouli et al. 2005). While

dramatic progress on the *RAD6* dependent pathway has been made to understand the response to genotoxic insults, the function of this pathway on fork integrity at natural replication barriers remain obscure.

It is believed that the choice of repair pathways is through competition by passive shunting based on the availability of repair enzyme (Gudmundsdottir et al. 2007). However, recent evidence suggests there is an active regulation control repair pathway choice. In yeast, the homolog search and strand invasion in G1 phase is prevented by blocking the activation of Mec1 and the loading of RPA and Rad51(Ira et al. 2004). This suppression of HR depends on CDK1 activity (Ira et al. 2004), and similar cell cycle regulation also occurs in mammalian. The interaction of BRCA2 and RAD51 is blocked by phosphorylation of BRCA2 by CDK (Esashi et al. 2005), representing one of the mechanisms of downregulation of HR in M phase and G1 phase. However, such cell cycle regulation is overcome when cells were irradiated (Esashi et al. 2005), indicating a multi-level regulation on the deployment of HR pathway.

Although, recombination is believed to be fairly accurate, recombination between inappropriate sequence partners can lead to translocations or other deleterious rearrangements and such events must be avoided. For example, the recombination machinery must follow stringent rules to preclude recombination between the many interspersed repeat elements in a mammalian genome (Waldman 2008). Another restriction of HR may be present in the haplophase during the sexual cycle when the homolog template is not available. Many protozoa, algae, fungi, mosses, and ferns maintain an alternation of generation with substantial development in both haploid and diploid (Mable and Otto 1998). However, little is known about how the cells sense the

existence of homolog copy and how the cells prevent recombination between interspersed repeat sequences.

In our study, using yeast as a model, I investigate the repair response under replication stress induced by *mcm4*$^{Chaos3}$ (Chapter 3). I find haploid and diploid mutant have adopted different repair pathways. The haploids use the *MGS1*- and *RAD6*-dependent pathways to resume stalled forks without engendering GIN, while in diploids, stalled forks collapse to provide substrates for error prone repair by HR at interspersed repeat sequences. This dichotomous choice is due to neither the availability of different repair enzymes nor the *MAT* heterozygosity, but ploidy itself. Therefore, our study indicates the haploid and diploid repair pathways do not randomly compete, and underlines the importance of the coupling between ploidy and the appropriate DNA repair pathway for genome stability maintenance.

***Meiotic recombination and pachytene checkpoint***

Recombination is an ancient phenomenon, predating its higher-level manifestation in sexual reproduction by 3 billion years of stasis (Cavalier-Smith 2002a; Lehman 2003). The recombination machinery was adopted later for meiosis probably by co-opting the consequence of crossing over to ensure disjunction during ploidy reduction (Cavalier-Smith 2002b). Crossing over is critical for the proper segregation of homologous chromosomes during the first meiotic division. The chiasmata formed by crossing over events physically tether chromosome homologs, thus helping to keep them attached through diplonema when interhomolog cohesins are removed, until Metaphase I. Organisms ensure that each homolog contains at least 1 crossover per chromosome either by having a large number of crossovers/meiosis, or by using interference to ensure that a limited number of crossovers are distributed, in a non-random fashion, to all chromosomes. Failure of a chromosome pair to undergo at

least 1 crossover can result in both segregating to the same daughter cell, leading to aneuploidy.

To drive homologous chromosome synapsis and ultimately crossovers, double strand breaks (DSBs) are genetically-induced in leptonema, which stimulate homologous recombination repair. Recombination repair is coupled with synapsis in budding yeast and mammals. While our knowledge on the assembly and nature of recombination machinery is extensive, little is known about the disassembly of recombination intermediates and recruitment of DNA replication machinery during recombinational repair.

While more than 200 DSBs are created during mouse meiosis, only 20-25 are resolved to be crossovers (CO) in mice. Evidence from *S. cerevisiae* indicates CO and non-crossover (NCO) pathways are distinct (Allers and Lichten 2001); they are likely to have different recombination intermediates, and are dependent upon different proteins (Hunter and Kleckner 2001; Borner et al. 2004). Mice also appear to have independent CO vs. NCO recombination pathways (Guillon et al. 2005). As in yeast, both require SPO11-induced breaks, but only the CO pathway requires MLH1. Both types of recombinant products are formed by mid-late pachynema. So far no protein has been identified to be specifically involved in NCO pathway in mammals, and little is known about how the two pathways are regulated.

Another essential aspect of HR in meiosis is that recombination must occur between homologous chromosomes (interhomolog recombination, or IH), rather than sister chromatids (intersister, or IS), to drive synapsis and crossing over. In yeast, there is a strong preference for IH recombination. Interestingly, elements of the DNA damage checkpoint and SC (axial element proteins Red1 and Hop1) combine in DSB repair partner choice. Recently, Carballo et al showed that the *S. cerevisiae* axial

element protein Hop1 is phosphorylated by Tel1/Mec1 in response to meiotic DSBs (Carballo et al. 2008). This then stimulates phosphorylation of Mek1 (ortholog of mammalian CHK2) to stimulate Dmc1-mediated IH recombination. Deficiency of Hop1, or mutation of [S/Q]T phosphorylation sites, resulted in Dmc1-independent repair of DSBs by IS recombination. No mammalian proteins involved in partner choice are known, and there are no clear orthologs of either Red1 or Hop1.

Defects in recombination can preclude homologous chromosome pairing, leave unrepaired chromosome breaks, and cause aneuploidy by abrogating crossing over. To avoid such deleterious outcomes, many organisms have evolved surveillance systems ("checkpoints") to sense meiotic errors and eliminate cells containing unresolved defects (reviewed in Chapter4 (Li et al. 2008)). Despite the extensive work in yeast, the mechanisms of putative checkpoint control remain unknown in mammals, since no mutations have been identified that abolish it.

I reported that the mouse ortholog (*Trip13*) of pachytene checkpoint 2 (*PCH2*), an essential component of the synapsis checkpoint in *S. cerevisiae* (San-Segundo and Roeder 1999; Wu and Burgess 2006) and *C. elegans* (Bhalla and Dernburg 2005), is required after strand invasion for completing a subset of recombination events, but possibly not those destined as crossovers (Li and Schimenti 2007)(Chapter5). Surprisingly, I found no evidence for checkpoint function, suggesting different pachytene checkpoint mechanisms may be involved in different species (Li et al. 2008). Given the high similarity of PCH2 orthologs throughout the eukaryotic world, one or more essential functions of this protein must be conserved. Since TRIP13 does not exhibit checkpoint function in mice, I surmise that the TRIP13/PCH2 ancestral protein had a function in recombination that persists to the present. Recent additional evidence from budding yeast indicates that Pch2p also functions in recombination

(Borner et al. 2008). In summary, the cross-model organism approach sometimes yield divergent results. We need to be cautious in making generalization for all eukaryotes from observations in lower eukaryotes.

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# CHAPTER 2

## A Defective MCM Helicase Predisposes Yeast to Aneuploidy and Accelerated Proliferation


### Abstract

Aneuploidy (including gross chromosome rearrangements, whole chromosome gains and losses) and genomic instability (GIN) are hallmarks of cancer cells. However their sources and functions in tumorigenesis are unclear. *Mcm4*$^{Chaos3}$, which encodes a mutant subunit of the hexameric MCM helicase, was previously reported to cause GIN and predispose homozygous female mice to mammary adenocarcinomas. I found that homozygous diploid yeast carrying the equivalent mutation show G2/M delay and a dramatic increase in loss of heterozygosity (LOH). They are defective in replicating long terminal repeat (LTR) elements, and generate a hypermutable subpopulation with severely compromised growth. Through this hypermutable stage, offspring acquire distinct new traits such as accelerated proliferation (AP) and various types of aneuploidy. Although AP and aneuploidy are 100% correlated in this subpopulation, I provide conclusive evidence that mutations unrelated to aneuploidy are the cause of the AP phenotype. Our results show that in the *mcm4*$^{Chaos3}$ mutant replication defects at LTR regions combined with error prone repair are the source of GIN, which is the driving force for the acquisition of AP with aneuploidy as byproducts. This study distinguishes the effects of GIN and aneuploidy providing insights for their distinct roles in the evolution of cancers.

## Introduction

Aneuploidy, referring to numerical and structural chromosome aberrations, is a common feature of cancers (Mitelman F 2008). Aneuploidy is believed to contribute to tumorigenesis through a mechanism in which oncogenes are gained, tumor suppressor genes are lost, or oncogenic fusions are created at breakpoints (Cahill et al. 1998; Lengauer et al. 1998). However, decades of intense efforts in mapping chromosome rearrangement breakpoints in cancer cells failed to establish a causative relationship between aneuploidy and cancers (Heim and Mitelman 2008; Mitelman F 2008). A major difficulty in these animal studies is that it is impossible to trace the ancestral cell with the initiating oncogenic mutation and establish a subclonal phylogeny of somatic mutations acquired during clonal expansion in cancer development (Campbell et al. 2008). Two recent yeast and mouse studies challenge the prevailing notion that aneuploidy is the cause of cancer by constructing strains or primary cells bearing an extra copy of chromosome and by showing that these strains have a proliferative disadvantage (Torres et al. 2007; Williams et al. 2008). However, the argument could be made that certain types of aneuploidy in combination with aneuploidy tolerating mutations may confer proliferative advantage. Furthermore, proliferation advantageous aneuploidy acquired during the course of tumor progression may not be represented in these artificially constructed strains (Hernando 2008). The definitive demonstration of a causative relationship between aneuploidy and cancer is to follow cancerous traits after removing aneuploidy from cancer cells. So far, it is impossible to remove aneuploidy from cancer cells.

GIN, a hallmark of cancers (Loeb 2001; Rajagopalan et al. 2003), refers to the unfaithful transmission of genetic information from an ancestral precursor (Schar 2001). It is believed to be the cause of aneuploidy in tumors (Raptis and Bapat 2006;

Bayani et al. 2007). However, GIN alone is deleterious because most random mutations are harmful (Burhans and Weinberger 2007). Therefore, it has also been argued that GIN is a consequence rather than the initial driving force of tumorigenesis (Tomlinson and Bodmer 1999). Accelerated proliferation (AP), on the other hand, is a characteristic associated with the cellular transformation of cancer cells during tumor progression. There is no direct evidence that GIN promotes AP in normal proliferating cells, and little is known about the intermediate steps required to engender AP in cells with GIN. The causal relationships between GIN, aneuploidy and AP have been the focus of intense investigations among cancer biologists for decades without consistent evidence for a unifying hypothesis. In this study, using yeast as a model, I delineate the relationships of GIN, aneuploidy and AP.

The source of GIN in this study is a cancer susceptible allele, *Mcm4*$^{Chaos3}$, which was first identified in a forward genetic ENU mutagenesis screen for mice exhibiting chromosome instability (Shima et al. 2007a). Mcm4 is a subunit of the evolutionarily conserved heterohexameric MCM2-7 helicase, essential for replication initiation and elongation (Maine et al. 1984; Tye 1999; Forsburg 2004; Moyer et al. 2006). *Mcm4*$^{Chaos3}$ is a point mutation (F341I) located in a conserved region at the interface of neighboring subunits (Supplementary Figure1). Female mice homozygous for *Mcm4*$^{Chaos3}$ are highly prone to mammary tumors with a mean latency of 12 months, suggesting that *Mcm4*$^{Chaos3}$ has minimal deleterious effects on the animal as a whole and that tumor progression is a relatively slow process (Shima et al. 2007a). Furthermore, this ubiquitous effect of the MCM helicase seems to target only mammary tissues in female mice for cancer development in a defined genetic background. Thus, this homozygous *Mcm4*$^{Chaos3}$ mouse strain provides an excellent model for studying mammary tumor development in mammals targeted by a single mutation that alters the activity of a component of the DNA replication machinery.

Recent evidence suggests that the MCMs may also play a role in DNA repair (Shukla et al. 2005) and DNA damage response (Malumbres and Barbacid 2001; Cortez et al. 2004; Bailis et al. 2008). To investigate the molecular mechanisms that lead to GIN as a result of the *Mcm4*$^{Chaos3}$ mutation, we introduced the equivalent mutation into diploid yeast. Here, I show that defective DNA replication at LTR regions coupled with error prone recombination generates a hypermutable subpopulation of yeast cells that acquires new traits including aneuploidy and AP. I delineate the phylogeny of subclones derived during clonal expansion from an ancestral *mcm4*$^{Chaos3}$ cell and characterize the chromosome rearrangements of these subclones by comparative genomic hybridization (CGH). I show that neither aneuploidy nor the *mcm4*$^{Chaos3}$ mutation contributes to the maintenance of AP state. On the contrary, removal of aneuploidy from AP cells by genetic crosses further accelerates proliferation suggesting that aneuploidy confers proliferative disadvantage. More importantly, I show that mutations unrelated to aneuploidy are responsible for AP.

## Results

### The *mcm4*$^{Chaos3/Chaos3}$ diploid exhibits a *RAD9*-dependent G2/M delay

To investigate the mechanistic basis of GIN caused by *Mcm4*$^{Chaos3}$, we introduced the equivalent mutation F391I into diploid yeast strains (Shima et al. 2007a). I found that *mcm4*$^{Chaos3/Chaos3}$ yeast had a G2/M delay based on FACS analysis of log phase cells (Figure 1A). At 30°C, the doubling time of *mcm4*$^{Chaos3/Chaos3}$ (3.02±0.16 h) was longer than those of wild-type (2.05±0.06 h) and *mcm4*$^{Chaos3/+}$ (2.14±0.06 h). I observed that the proliferating mutant cultures had an increased proportion of large budded cells with one nucleus at the bud neck (Supplementary

**Figure 1.** The *mcm4*$^{Chaos3/Chaos3}$ mutant has a replication elongation defect. A) The mutant shows a G2/M delay that is Rad9 dependent and Mad2-independent. B) *mcm4*$^{Chaos3/Chaos3}$ shows synthetic growth defects with fork stabilizing mutations *mrc1Δ* and *tof1Δ*. Tenfold serial dilutions of each strain were spotted on complete medium plates and incubated at 37°C. C) The *mcm4*$^{Chaos3/Chaso3}$ *rad51Δ/Δ* double mutant shows synthetic conditional lethality at 37°. D) and E) *mcm4*$^{Chaos3/Chaos3}$ *rad51Δ/Δ* mutant was grown to log phase at 30°C and then shifted to 37°C for three hours. D) FACS profile shows that most cells are arrested with about 4C DNA. There is no cell cycle delay in *rad51Δ/Δ* single mutant (Data not shown). E) CGH analysis of the double mutant arrested at 37°C. Three under-replicated regions with >8-fold decrease in DNA were observed. Region 1 corresponds to *RAD51*, which is deleted in the mutant strain; this serves to validate the array sensitivity. Regions 2 and 3 correspond to locations that abut solo LTRs (Supplementary Fig 3).

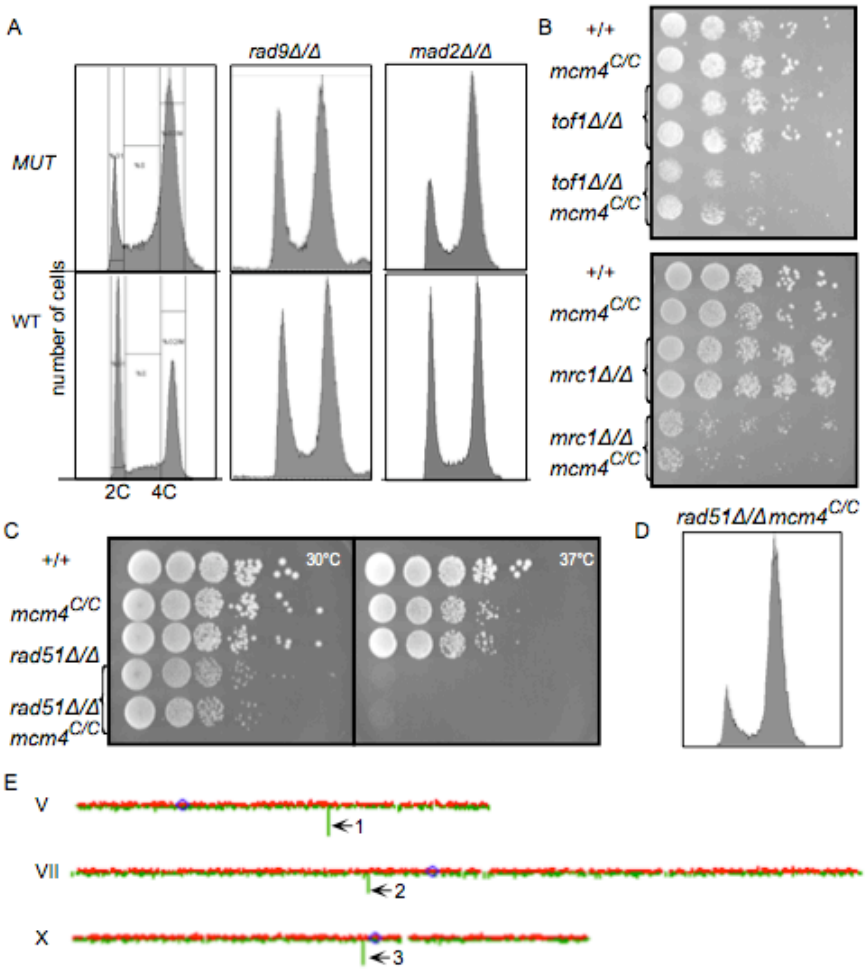

A
rad9Δ/Δ
mad2Δ/Δ
MUT
WT
number of cells
2C
4C
B
+/+
mcm4C/C
tof1Δ/Δ
tof1Δ/Δ mcm4C/C
+/+
mcm4C/C
mrc1Δ/Δ
mrc1Δ/Δ mcm4C/C
C
+/+
30°C
37°C
mcm4C/C
rad51Δ/Δ
rad51Δ/Δ mcm4C/C
D
rad51Δ/Δmcm4C/C
E
V
VII
X
1
2
3

Figure2 B,C,D), indicating a delay prior to anaphase. This G2/M delay is a checkpoint response triggered by DNA damage. Knocking out the DNA damage checkpoint protein Rad9 (Foiani et al. 2000) abolished the G2/M delay, while knocking out the spindle assembly checkpoint protein Mad2 (Hoyt 2001) had no effect (Figure1A). The *mcm4*$^{Chaos3}$ allele was slightly temperature-sensitive (ts) (Supplementary Figure2A). As in mice (Shima et al. 2007a), these defects are more severe in the yeast *mcm4*$^{Chaos3/\Delta}$ mutant, which has a doubling time of 3.72±0.15 h, suggesting that *mcm4*$^{Chaos3}$ caused a loss of function of MCM4. Interestingly, the phenotypes described for the *mcm4*$^{Chaos3}$ homozygous diploid are not observed in the haploid mutant (data not shown). Therefore, all of the experiments described in this paper are carried out in diploid cells.

**The *mcm4*$^{Chaos3/Chaos3}$ diploid shows a 100-fold increase in LOH due to hyper-recombination**

Loss of heterozygosity (LOH) is a major contributing event in cancer development and a product of GIN. To investigate whether the *mcm4*$^{Chaos3}$ allele causes GIN in yeast, I measured the LOH frequency of *CAN1* with respect to *HOM3* on the left arm of chromosome V (Hartwell and Smith 1985). Almost all detected LOH events were due to mitotic recombination. There was little difference in the frequency between *MCM4*$^{+/+}$ ($2.12 \pm 0.11 \times 10^{-5}$) and *mcm4*$^{Chaos3/+}$ ($3.04 \pm 0.73 \times 10^{-5}$) yeast but the frequency in *mcm4*$^{Chaos3/Chaos3}$ ($2.60 \pm 1.60 \times 10^{-3}$) was about 100-fold elevated over that of the wild type. This frequency is much higher than any DNA damage checkpoint, recombination, or repair mutants reported to date (Klein 2001; Craven et al. 2002). Therefore, the DNA damage that triggers the G2/M delay is likely induced by *mcm4*$^{Chaos3}$ directly rather than caused by the failure of *mcm4*$^{Chaos3}$ to repair spontaneous DNA damages. The greatly stimulated recombination rate and

G2/M delay suggests that the *mcm4*$^{Chaos3/Chaos3}$ mutant has an intact repair and checkpoint system, and that the damage is probably caused by its replication defect.

**_mcm4_$^{Chaos3/Chaos3}$ is defective in replicating solo LTR elements**

The MCM helicase has been shown to migrate with the elongation fork during DNA replication (Aparicio et al. 1997). To investigate if *mcm4*$^{Chaos3}$ mutant has compromised replication forks, I constructed double mutants of *mcm4*$^{Chaos3}$ with *mrc1Δ* or *tof1Δ*. Mrc1 and Tof1 are replication fork stabilization proteins that are loaded onto DNA shortly after initiation and travel with the replication fork (Katou et al. 2003). The synergistic growth defects of *mcm4*$^{Chaos3}$ with *mrc1Δ* and *tof1Δ* (Figure 1B) suggest that *mcm4*$^{Chaos3}$ leads to replication fork defect that require fork stabilization. Considering the 100-fold increase in mitotic recombination observed in the *mcm4*$^{Chaos3}$ strain, recombination mediated replication is a likely mechanism for repairing the fork defects in *mcm4*$^{Chaos3}$ mutant. Accordingly, I placed *mcm4*$^{Chaos3}$ into a recombination-deficient background (*mcm4*$^{Chaos3/Chaso3}$ *rad51Δ/Δ*). The double mutant showed synthetic lethality at 37°C (Figure 1C) arresting with about 4C DNA (Figure 1D). This result indicates that homologous recombination (HR) is indispensable for repairing DNA damage induced by *mcm4*$^{Chaos3}$.

DNA replication stress has been shown to have a more dramatic effect on particular regions of mammalian genomes such as the common fragile sites (CFSs) (Glover et al. 2005). The notion is that replication forks are more likely to stall or collapse at vulnerable regions of the genome (Arlt et al. 2004; Durkin and Glover 2007). By preventing their repair, I should be able to map the CFSs in yeast where replication forks stall. I took advantage of the ts phenotype of *mcm4*$^{Chaos3/Chaso3}$ *rad51Δ/Δ* cells to map potential fork barrier zones, which should be under-replicated at the restrictive temperature relative to other regions in the genome. Using

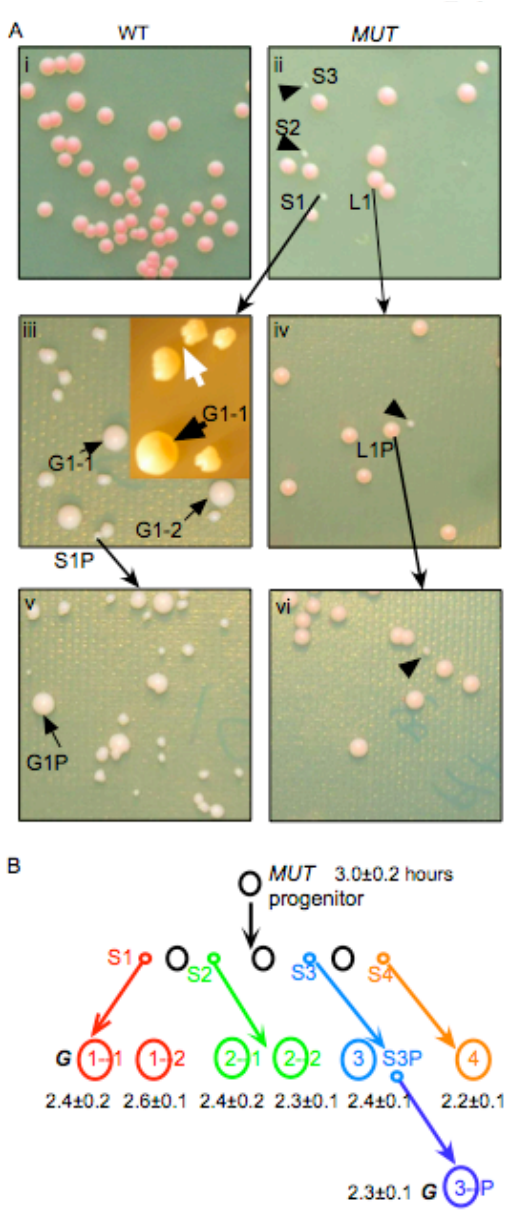
comparative genomic hybridization (CGH), I saw under-replication of two ~290 bp regions contiguous to solo LTR elements on chromosomes VII and X, respectively (Figure 1E, Figure 2 and Figure 3 Supplementary Figure 3). I believe the number of fork barrier zones is greatly underestimated in this experiment because of the exclusion of repetitive sequences in the Agilent microarray used in the CGH. These results suggest that the *mcm4*$^{Chaos3}$ mutants are defective in replicating LTR-enriched regions probably as a result of a compromised MCM complex. Such fork defects may trigger the observed G2/M delay and stimulate mitotic recombination, leading to LOH.

In *S. cerevisiae*, there are about 300 Ty related elements each flanked by LTRs clustered in about 30 - 40 locations (Gabriel et al. 2006). In addition, there is an order of magnitude more solo LTR elements (Gabriel et al. 2006). Altogether, these repetitive sequences represent about 3% of the genome. Tys and LTRs have been shown to be hotspots for translocation (Dunham et al. 2002; Lemoine et al. 2005; Argueso et al. 2008). It was unclear whether they are intrinsic fragile sites or preferential sites of repair for randomly distributed damage. Our results suggest that LTRs more likely than whole Ty elements pose as barriers under replication stress. Repair of damages by ectopic recombination at these repetitive sequences is likely to result in translocations and dicentric chromosomes that initiate cycles of GIN (Admire et al. 2006).

**A subpopulation of *mcm4*$^{Chaos3}$ cells form minute colonies**

Possibly a consequence of GIN, *mcm4*$^{Chaos3/Chaos3}$ showed 40% decreased viability (Supplementary Figure 4A) compared to wild type and gave rise to a subpopulation that formed minute colonies (Figure 2A,ii). Whereas colonies of wild type yeast are uniform in size, I found that mutant yeast formed variably-sized

**Figure 2.** The *mcm4*$^{Chaos3/Chaos3}$ mutant generates a subpopulation of genetically unstable cells. A) The mutant produces heterogeneous offspring (i) Wild type cells produce uniform sized colonies. (ii) The mutant produces heterogeneous-sized colonies. The arrowheads point at representative minute colonies, S1, S2 and S3. Long black arrows indicate the lineage of colonies that were analyzed. L1 and S1 are a large and a minute colony derived from a streak of a large colony of the mutant. (iii) and (iv) are colonies derived from S1 and L1 respectively. (iii) heterogeneous colony morphologies include giant (G1-1, G1-2), serrated (white arrow) and minute (S1P) colonies. The inset is a magnification of the heterogeneous colonies. (v) S1P gives rise to heterogeneous colonies including giant colonies such as G1P. (vi) Large colonies (L1P) consistently give rise to both large and minute colonies. B) The lineage of strains presented in figures 2 and 3. Strains derived from the same ancestral small colony are color-coded. The numbers next to the strains are the doubling time (hour). S = minute, L = large, G = giant, P = progeny.


A
WT
MUT
i
ii
S3
S2
S1
L1
iii
G1-1
G1-1
G1-2
S1P
iv
L1P
v
G1P
vi
B
MUT 3.0±0.2 hours
progenitor
S1
S2
S3
S4
G 1-1
1-2
2-1
2-2
3
S3P
4
2.4±0.2
2.6±0.1
2.4±0.2
2.3±0.1
2.4±0.1
2.2±0.1
2.3±0.1 G 3-P

colonies that showed a bimodal distribution (Supplementary Figure 4C). This bimodal distribution of large and minute colonies was reproduced upon replating of the large colonies (Figure 2A,ii, L1 and 2A,iv, L1P). Replating of the minute colonies gave rise to a dramatically heterogeneous distribution (Figure 2A,iii), including minute, serrated (white arrow), and giant colonies (G1-1 & G1-2). The minute S1P retained the ability to produce heterogeneous offspring including giant colonies (Figure 1Bv, G1P) upon restreaking. The serrated morphology is typical of yeast cells that are continuously giving rise to offspring with different viabilities and growth rates (Admire et al. 2006). A key observation is that giant colonies readily emerge from a single restreaking of minute colonies, but rarely from the direct restreaking of large colonies as if an intermediate step involving hypermutagenesis is required for this transition.

**Progeny of minute colonies acquire new traits**

The giant colonies were curious because of their size and smooth morphology, traits indicative of cells having a relatively shorter doubling time and more stable genome than their progenitors from the minute colonies. An obvious explanation for their emergence is that secondary genetic events must have overcome the genetic instability of the progenitor cells. To investigate these secondary genetic events, seven giant colonies with lineages traced to a single founder cell were characterized (Figure 2B). Consistent with their colony size, they all had shorter doubling times than their ancestral progenitor (Figure 2B). Other than the common AP phenotype, each strain exhibited additional distinct new traits. Some have viability that surpasses that of the ancestral progenitor, while some have decreased viability (Figure 3A). FACS analysis indicated that these strains still maintained a near-diploid DNA content, and some of them had a less pronounced G2/M delay than their ancestral progenitor

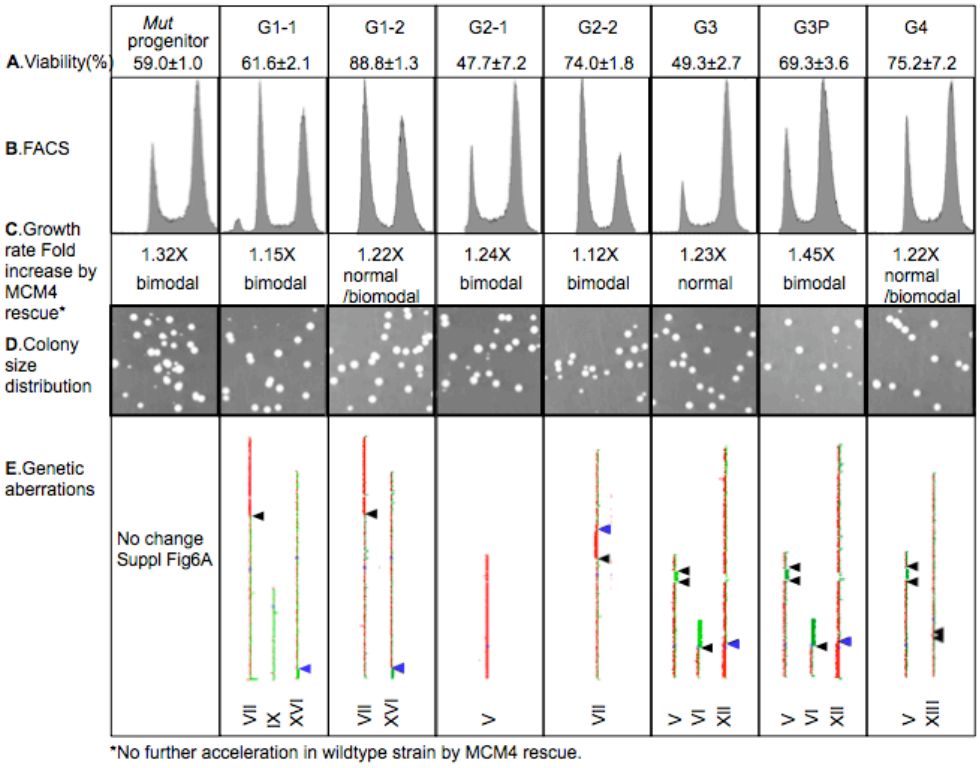



**Figure 3.** New traits acquired by cells of giant colonies. Viability (A), FACS profiles (B), fold increase in growth rates, with or without wildtype *MCM4* on a *CEN* plasmid (C), colony size distribution (D) and aneuploidy (E). CGH in E: yellow indicates approximately equal amounts of hybridization between mutant and wild type DNA; green indicates approximately 2-fold reduction and red approximately 1.5-2-fold increase in mutant. Arrowheads (black, Ty; blue, solo LTR) represent the breakpoints of translocations, amplifications or deletions. Detailed characterization of colony size distributions, and genetic aberrations are shown in supplementary Fig 4B & 5, respectively.

(Figure 3B). The distribution of colony size also varied among these strains (Figure 3D, Supplementary Figure 4C). The distinct new traits of the giant colony forming cells suggest that these traits are acquired independently and that AP cells from independent giant colonies may result from different underlying mechanisms. Since giant colonies were formed only when cells from minute colonies were removed from the competitive selective pressure of the main population, the acquisition of new traits seems to be mainly driven by GIN.

To investigate whether the secondary genetic events require *mcm4*$^{Chaos3}$ to maintain the AP state, I transformed a wild-type *MCM4* allele into these fast-proliferating strains. In the *mcm4*$^{Chaos3}$ progenitor strain, the growth defect was partially rescued by the wild-type *MCM4* allele (Figure 3C). Proliferation rates of AP strains (Figure 3C) but not wild type strain (Supplementary figure 4B) were further accelerated by *MCM4*, suggesting that some other genetic events are responsible for the AP independent of the *mcm4*$^{Chaos3}$ background. Therefore, unlike oncogene-induced AP (Felsher 2004), the mutation that initiates GIN is not required to maintain the AP state.

**Fast-proliferating *mcm4*$^{Chaos3/Chaos3}$ strains are associated with various types of aneuploidy.**

To investigate the effects of *mcm4*$^{Chaos3}$ on genome integrity and the genetic events associated with AP, I analyzed the karyotypes of these seven fast-proliferating strains by CGH and, when translocations were indicated, by PCR analysis. Each strain had a unique spectrum of genetic aberrations, including translocations, segmental duplications and deletions, whole chromosome gains or losses, and gene amplifications (Figure 3E). I did not observe a common chromosomal aberration that could be identified as a defining primary genetic change responsible for the fast-

proliferation phenotype. I found that the breakpoints of all of the chromosomal rearrangements were associated either with Ty or solo LTR elements (Figure 3E arrowheads, Supplementary Figure 5), supporting the idea that defective replication at LTR regions is the underlying mechanism for GIN.

**The subpopulation of minute colonies is hypermutable**

To investigate when giant colonies acquired aneuploidy during their clonal expansion from the minute progenitors, I compared the karyotypes of pairs of AP strains each derived from a common minute progenitor. Giant colonies G1-1 and G1-2, both derived from colony S1 (Figure 2B), shared a common translocation of a segment of the right arm of chromosome VII to the left arm of chromosome XVI (Figure 3E, Supplementary Figure5A), suggesting that this particular translocation event may have occurred very early within the S1 colony. However, G1-1 also had a loss of Chr IX, an event not shared by G1-2, suggesting that Chr IX was lost later during the formation of the minute colony, S1. This result demonstrates that the subpopulation of $mcm4^{Chaos3/Chaos3}$ cells that form minute colonies are genetically unstable, a property that is consistent with the heterogeneous morphologies of colonies generated by these cells upon restreaking (Figure 2A,iii, v).

**The hypermutable subpopulation is unlikely generated by aneuploidy**

The comparison of G1-1 and G1-2 indicates that aneuploidy is acquired during clonal expansion. However, it is unclear whether aneuploidy is driving the generation of this subpopulation that forms minute colonies. G2-1 and G2-2, derived from S2 shared no common gross chromosomal aberration (Figure 3E, Supplementary Figure 5B) indicating that the observed aberrations must have been generated after the emergence of the S2 founder cell. Therefore, aneuploidy *per se* is unlikely the driving

force for the formation of this hypermuatable subpopulation. These results argue that GIN came first as the driving force and aneuploidy came later as a consequence.

**Aneuploidy is not responsible for AP**

So far, I observed 100% association of AP and aneuploidy. To investigate whether and which specific chromosome aberration may be responsible for AP, I carried out two types of analysis. First, I correlated aneuploidy and proliferation based on lineage. If aneuploidy were associated with AP, slow-growing siblings of fast growers would not be aneuploid or would have distinct genetic aberrations. The giant colony G3's minute sibling (S3P) was streaked further to generate G3P because S3P was too unstable for karyotype analysis (Figure 2B). Remarkably, I found that G3 and G3P share multiple identical genetic aberrations (Figure 3E). These aberrations unlikely arose independently and more likely arose in S3, the slowly proliferating minute progenitor cells of G3 and S3P. Therefore the progenitor cell of S3P must have already acquired the aneuploidy that is associated with AP in G3 and G3P, suggesting that aneuploidy is unrelated to AP. Despite their identical aneuploidy, G3 and G3P have distinctly different viability, cell cycle profiles and colony sizes (Fig 3, Supplementary Figure 4C). Such traits presumably are caused by genetic mutations distinct from the shared chromosome alterations and are acquired independently during clonal expansion of their respective minute progenitors.

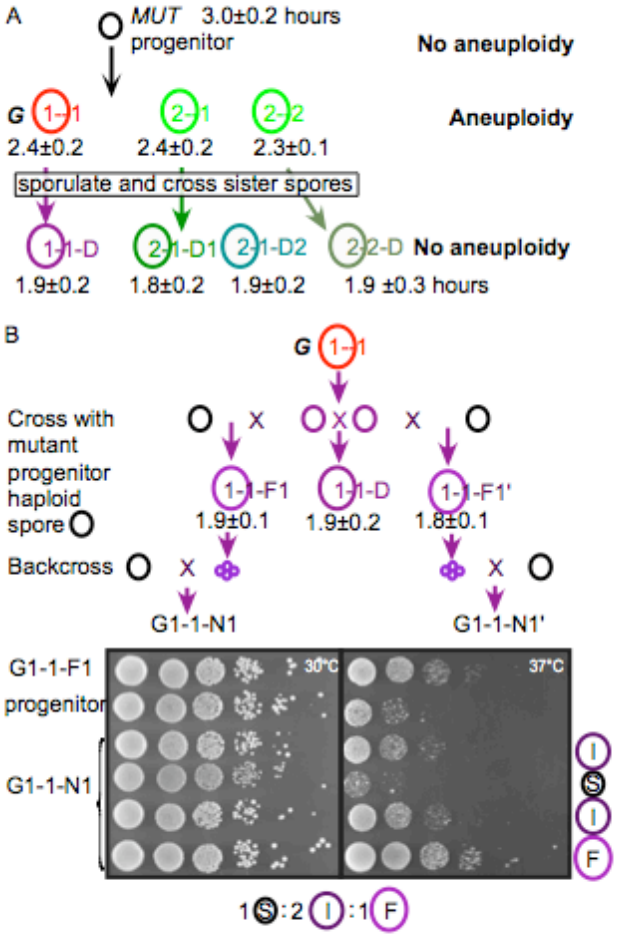
A second approach to investigate the effect of aneuploidy on AP was to remove chromosome aberrations from AP strains by sporulating G1-1, G2-1, G2-2 and mating sister spores (Figure 4A). I then performed CGH on the derivative diploids. G1-1D, G2-1D-1, G2-1D-2 and G2-2D showed no aneuploidy (Supplementary Figure 6) but all exhibited even shorter doubling times than their giant parent strains (Figure 4A). This result confirms that CGH detectable aneuploidy is not required for

accelerated proliferation. Rather, other secondary mutations contribute to AP. Therefore, aneuploidy is a byproduct of the GIN-driven transition of cells from a normal to an AP state. More importantly, I found no evidence for chromosome aberrations having an enhancing effect on proliferation rates. On the contrary, I found that they retard proliferation rates.

**Mutations associated with and responsible for the AP phenotype**

If aneuploidy is not the cause of the AP phenotype, then what are the mutations that cause AP? The parents of the AP strain (G1-1D) were backcrossed with the progenitor *mcm4*$^{Chaos3}$ strain (Fig 4B) for the AP phenotype. The resulting diploids (G1-1-F1 and G1-1-F1') in a heterozygous background for the secondary mutations also show AP (Figure 4B). These results indicate the AP phenotype in G1-1 is dominant. In principle, one should be able to observe Mendelian segregation of the mutation(s) linked to AP by tetrad analysis if the AP phenotype is dominant and if the AP phenotype is determined by one or two alleles. G1-1-F1 and G1-1-F1' were sporulated. Three tetrads of G1-1-N1 and G1-1-N1' were analyzed further for proliferation proficiency by mating to the progenitor *mcm4*$^{Chaos3}$ strain. Instead of measuring growth rates at 30°C, the segregation pattern of the AP phenotype is best demonstrated by plating the resulting diploids on YPD plates at 37°C. The AP phenotype segregated 1:2:1 in all three tetrads examined (Figure 4B, and Supplementary Figure 6F) suggesting that two alleles in G1-1-N1 and G1-1-N1' constituted the AP phenotype. I do not know if these alleles are identical for G1-1-N1 and G1-1-N1'. If so, LOH may play a role in the homozygosity of these alleles in G1-1. This genetic approach may be applied to individual AP strains to estimate the number of alleles that contribute to the AP phenotype.

**Figure 4**. Mutations unrelated to aneuploidy contribute to accelerated proliferation. (A) Cells from giant colonies were sporulated and sister spores were mated. Those with AP phenotype were devoid of aneuploidy (confirmed by CGH, see Suppl. Figure 6) and show even more enhanced proliferation rates. Doubling times of the resulting diploids are shown. (B) The AP phenotype is dominant and segregates 1:2:1 in a tetrad. The parents of G1-1-D were crossed with progenitor *mcm4*$^{Chaos3}$ strain to form G1-1-F1 and G1-1-F1' which were sporulated for tetrad analysis. Tetrads were backcrossed to the progenitor *mcm4*$^{Chaos3}$ strain for AP phenotype. The growth rates of the resulting diploids from one tetrad of G1-1-N1 were compared by plating on YPD plate at 30°C and 37°C. Additional tetrads are shown in supplementary Fig 6F. F = fast, I = intermediate, S = slow.


A
MUT 3.0±0.2 hours
progenitor
No aneuploidy
G 1-1
2.4±0.2
2-1
2.4±0.2
2-2
2.3±0.1
Aneuploidy
sporulate and cross sister spores
1-1-D
1.9±0.2
2-1-D1
1.8±0.2
2-1-D2
1.9±0.2
2-2-D
1.9 ±0.3 hours
No aneuploidy
B
G 1-1
Cross with mutant progenitor haploid spore
1-1-F1
1.9±0.1
1-1-D
1.9±0.2
1-1-F1'
1.8±0.1
Backcross
G1-1-N1
G1-1-N1'
G1-1-F1 progenitor
G1-1-N1
30°C
37°C
I
S
I
F
1S:2I:1F

## Discussion

### *The effects of Mcm4$^{Chaos3}$ in mice are recapitulated in yeast.*

In this study, I have shown that a mutation in *MCM4* that predisposes mice to mammary adenocarcinomas also predisposes yeast to AP. There are other striking similarities between the mouse and yeast mutants (Table 1), indicating that the effects of *Mcm4$^{Chaos3}$* in mammals are recapitulated in yeast. This study serves as an excellent example that the molecular basis of complex diseases can be dissected in a simple model organism such as yeast, and that the information extracted from yeast may be used to guide mammalian and clinical studies.

### *DNA replication defects are the cause of GIN*

Although DNA replication defects have been hypothesized to play a direct role in cancer development (Schar 2001), evidence for this hypothesis is lacking. Here, I have shown that an amino acid change at the interface between Mcm4 and its neighboring subunit of the MCM helicase (Supplementary Figure1) causes a replication elongation defect that leaves the regions enriched for LTR elements at risk for replication. The precise locations of the unreplicated regions observed in this study (Figure 1E, Suppl Figure 3) suggest that LTRs directly pose a challenge for DNA fork migration in a manner analogous to fragile sites observed in mammalian chromosomes. A likely sequence of events supported by studies of the archael MCM helicase carrying the equivalent *mcm4$^{Chaos3}$* mutation is that the MCM helicase has reduced processivity due to instability of the complex (unpublished results), resulting in the pausing of replication forks at sequences that act as fork barriers to the helicase. I have devised an approach to systematically map replication fork barriers by blocking the repair pathway that rescues stalled forks. Similar strategies could be applied to other organisms.

**Table 1. Phenotypic similarities between *mcm4*$^{Chaos3}$ yeast and *Mcm4*$^{Chaos3}$ mice**

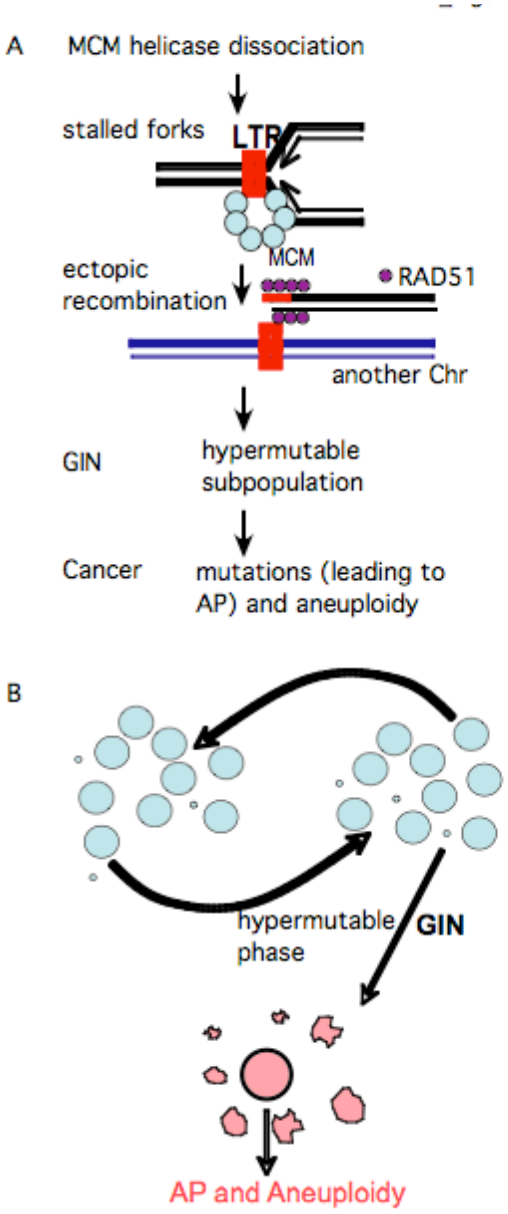
| Yeast | Mice (Shima et al. 2007a; Shima et al. 2007b) |
|---|---|
| G2/M delay | G2/M delay in *Mcm4*$^{C/C}$ MEFs and developmental lethality in *Mcm4*$^{C/\Delta}$ mice |
| Defective in replicating certain chromosomal sequences | Embryonic fibroblasts highly susceptible to chromosome breaks under replication stress |
| Elevated DNA damage requiring recombinational repair; 100 fold increase in mitotic recombination | 20-fold increase in frequency of micronuclei in erythrocytes, likely representative of elevated DSBs |
| Predisposition to AP | 80% of females acquire aggressive mammary tumors |
| Particular chromosome abnormalities in individual fast proliferation strains. | Different segmental aneuploidies in independent tumor cell lines (detected by array CGH; unpublished results) |

I believe that this replication elongation defect is the underlying cause of GIN in the *mcm4* mutant. Repair of stalled forks at LTR most likely are carried out by homologous recombination between LTRs either as solo elements or LTRs of Ty elements since participation of Rad51 is essential for survival of the $mcm4^{Chaos3}$ mutant (Figure 5A). The replication defects stimulated HR dependent repair to >100 fold over the normal rate. The effect of this hyper recombination at LTRs can be discerned in the footprints of the GCRs generated in the fast proliferating cells. Translocations, deletions and amplifications in these cells all have breakpoints at LTRs or Ty elements flanked by LTRs. I envision that hypermutable cells are generated when cells undergo ectopic recombination (Fig 5A). This scenario may be analogous to the expression of fragile sites in human (Glover et al. 2005). Ectopic recombination is highly mutagenic, which may account for the large variety of chromosome aberrations observed in the fast-proliferating strains. I believe that the DNA replication defect caused by $mcm4^{Chaos3}$ is the source of GIN that predisposes yeast to AP and mice to cancer.

***The hypermutable phase as an intermediate step to acquire new beneficial traits***

The classical view for the relationship between GIN and cancer is that only cells with subtle GIN undergo tumorigenesis (Cahill et al. 1999) because cells with severe GIN are eliminated by apoptosis or survival pressure. In this study, I find that only the hypermutable cells with compromised growth can ultimately generate fast growers when given the opportune environment to propagate without survival pressure. This observation suggests that GIN alone in the absence of survival pressure is sufficient to generate fast growers. In contrast, hypermutable subpopulations within the main cell population do not give rise to fast proliferating cells directly probably

**Figure 5.** Proposed mechanisms for the predisposition of *mcm4*$^{Chaos3/Chaos3}$ diploids to accelerated proliferation. A) A defective MCM helicase pauses and dissociates at LTRs which are barriers to the helicase at replication forks. HR repair using the homologous chromosome as template rescues most DSBs of stalled forks. At a certain frequency, fork damages are rescued by ectopic HR repair, resulting in GIN which fuels mutagenesis that generates aneuploidy as well as other mutations. Mutations other than aneuploidy are the cause of the AP phenotype. B) Cell culture undergoes self-renewal through continuous replenishing of genetically unstable cells that are outcompeted by healthier cells. However, such genetically unstable cells once removed from the survival pressure of the main population will go through cycles of genomic instability to generate cells that overcome the proliferative disadvantage of GIN to achieve the AP state.


A
MCM helicase dissociation
stalled forks
LTR
MCM
ectopic
recombination
RAD51
another Chr
GIN
hypermutable
subpopulation
Cancer
mutations (leading to
AP) and aneuploidy
B
hypermutable
phase
GIN
AP and Aneuploidy

because survival pressure weeds out the hypermutable cells that have a growth disadvantage (Fig 5B). As a result, the main population undergoes self-renewal for generations without apparent change to the proportion of cells that form large (genetically relatively stable) and minute (genetically unstable) colonies. This explanation may address why the *Mcm4*$^{Chaos3}$ mutation has little effect on the whole animal in mice and that its effects are only observed in target tissues that may somehow provide the safe haven for the survival of hypermutatble cells and the evolution of AP cells.

The existence of a hypermutable phase with severe growth defects during the development of AP cells reconciles with the long lasting debate about the cause and effect of GIN. Although GIN alone is deleterious (Burhans and Weinberger 2007), given a situation when survival pressure is alleviated, cells with GIN are able to slowly accumulate beneficial mutations that eventually can overcome the deleterious effects of GIN. Such a hypermutable stage that escapes survival pressure have been hypothesized to exist in early tumorigenesis (Sieber et al. 2003; Hernando 2008). Our study provides direct evidence for the existence and necessity of such a hypermutable stage for the adaptation of cells that ultimately achieve a high proliferative capacity.

**Causative relationship between GIN, aneuploidy and cancers**

GIN and aneuploidy are often viewed conceptually as being indistinguishable in that they both play causative roles in cancer evolution. In this study, I showed that GIN and aneuploidy are distinguishable in nature and function. GIN is the driving force for the acquisition of new genetic traits, some of which are discernable as cancer-promoting traits, in a manner similar to oncogene activation. However, unlike oncogene (Felsher 2004), the *chaos3* mutation that initiates GIN is not required to maintain the AP state. The irrelevance of aneuploidy in AP was demonstrated in two ways. First, sibling colonies (G3 and S3P) that differ dramatically in proliferation rates

(minute and giants) (Fig 2B) inherit the same genetic aberrations from their minute progenitor S3 (Fig 3E). Second, removal of aneuploidy from a fast proliferating strain further accelerates the proliferation rate (Fig 4A). The causative relationship between the replication stress at replication forks, GIN, aneuploidy, other mutations and AP is shown in Fig 5A. Based on our model, abnormal chromosomal contents characteristic of cancer cells can be nothing more than the baggage that comes with tumorigenesis and have no bearing on the cancerous state of tumors. Our finding is consistent with the artificially constructed chromosome gain mutants reported for yeast and mice (Torres et al. 2007; Williams et al. 2008) and extends to other types of GCRs including chromosome loss, translocations, segmental duplications and deletions as well as gene amplifications. I cannot rule out the possibility that by conferring proliferative disadvantage, aneuploidy may provide the bottleneck for the selection of aneuploidy tolerating and proliferation promoting mutations that is an integral part of tumorigenesis.

In summary, I showed that a DNA replication defect is the source of GIN, evident as hyper-recombination, which stimulates LOH as well as aneuploidy. I found that GIN and aneuploidy, despite their causal relationship, play distinct and separate roles in promoting the AP state: GIN is the driving force that is instrumental in generating mutations that bring cells to the AP state. Aneuploidy, like AP, is one of the consequences of GIN. I found that neither the *mcm4*$^{Chaos3}$ mutation nor aneuploidy is required to maintain the AP state. Rather, yet-to-be mapped mutations unrelated to aneuploidy are responsible for the AP state. Our findings in yeast bring insight to the roles of GIN and aneuploidy in tumorigenesis. I am poised to identify the mutations that underlie AP in yeast and to test their roles in cancer development in mice.

## Materials and Methods

*Yeast strains*

Isogenic diploid W303 yeast strains $mcm4^{+/+}$, $mcm4^{+/Chaos3}$, $mcm4^{+/\Delta}$, $mcm4^{Chaos3/Chaos3}$ and $mcm4^{Chaos3/\Delta}$ were constructed as described (Shima et al. 2007a). Strains used in this study are listed in Supplemental Table 1.

*Flow cytometric analysis*

Approximately 1 x $10^7$ cells were collected from log-phase cultures and processed as described (Clarke et al. 2001). DNA was stained with Sytox Green (Molecular Probes, Eugene, OR) and profiles were analyzed using a Becton Dickinson (San Jose, CA) LSR II with a 530/30BP channel filter and BDFACSDiVa software Becton Dickinson (San Jose, CA).

*Growth curve and doubling time*

Saturated cell cultures were diluted 25 X in complete medium and then grown at 30°C for 4 hours to mid-log phase. The absorbance at 600nm was measured every half hour for 5 hours. The growth rates and doubling times were calculated during exponential growth. For each experiment where doubling times of different strains are compared, all strains were processed simultaneously in at least two independent trials to yield variations in doubling times of less than 0.1 hr.

*Cell viability and colony size distribution*

Cell viabilities were measured by first counting log phase cells in a hemacytometer before plating in triplicate on YEPD and counting visible colonies after 3 days of growth at permissive temperatures. Colony sizes were quantified by ImageJ, and histograms were plotted by Excel.

*Mitotic recombination assay*

A standard assay for measuring mitotic recombination and chromosome loss was used(Hartwell and Smith 1985). The test strain was heterozygous for mutations in *CAN1* and *HOM3*, two markers located on opposite arms of chromosome V. The haploid strain with the *can1* mutation was resistant to canavanine ($Can^r$) and the *hom3* strain was auxotrophic for threonine ($Thr^-$). Heterozygous diploid strains were $Can^s$ and $Thr^+$. Mitotic recombination was scored by the $Can^r$ $Thr^+$ phenotype. Over 90% of the $Can^r$ strains scored were $Thr^+$.

*Comparative genomic hybridization (CGH) microarray*

Genomic DNA was prepared, sonicated and labeled based on the protocol from the Dunham lab (Torres et al. 2007). DNA from the experimental strain was labeled with Cy3 nucleotide, and DNA from wild-type strain was labeled with a Cy5 nucleotide. The two DNA samples were mixed and hybridized to Yeast Whole Genome ChIP-on-chip Microarray from Agilent (290 nt resolution, 4 x 44K slide format, which contains ~85% of the non-repetitive portion of the yeast genome catalog #G4493A). Arrays were then washed according to the Agilent SSPE wash protocol, and scanned on an Agilent scanner or Axon 4000B microarray scanner. The image was processed using the default settings with Agilent Feature Extraction software or GenePix Pro 6.0. All data analysis was performed using the resulting log2 ratio data, and filtered for signals that are 2.5-fold above background in at least one channel. Chromosome translocations are confirmed by PCR analysis.

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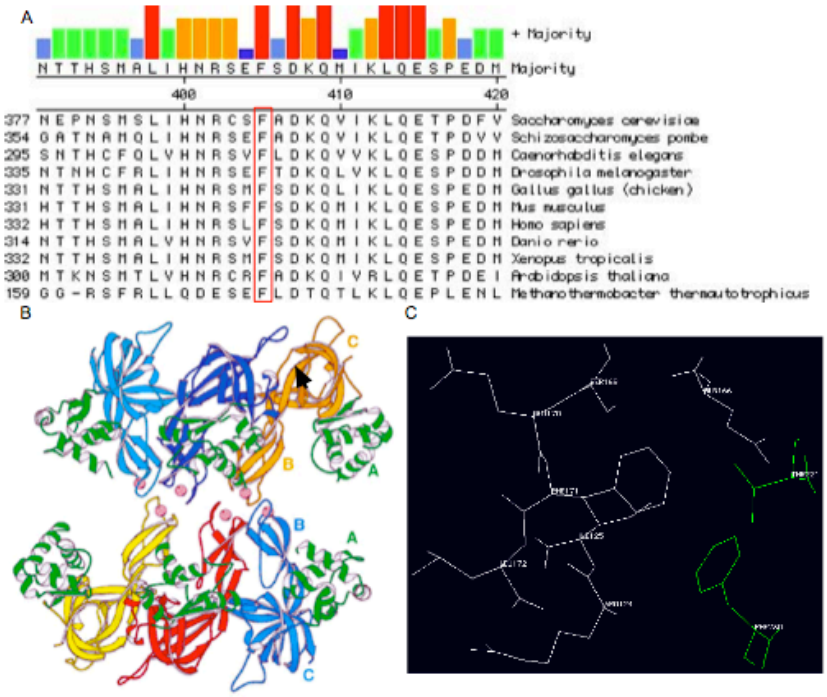
## APPENDIX

### Supplemental Table 1. Strain list

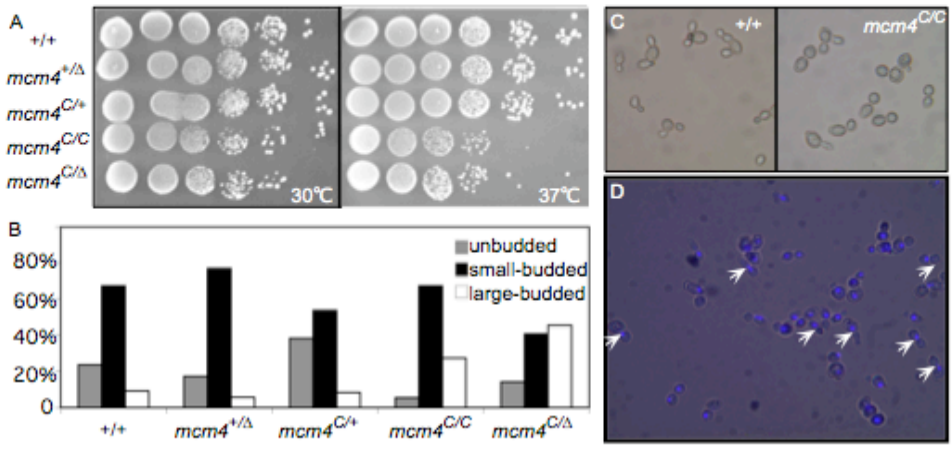
| Strain | Genotype | Source |
|---|---|---|
| W303 | MATa/MATa *ade2/ade2 his3/his3* | |
| (diploid) | *leu2/leu2 trp1/trp1 ura3/ura3 can1/can1* | |
| XLY495 | *mcm4*$^{Chaos3/Chaos3}$ | This lab |
| XLY494 | *mcm4*$^{Chaos3/+}$ | This lab |
| XLY506 | *mcm*$^{+/\Delta}$ | This study |
| XLY507 | *mcm4*$^{Chaos3/\Delta}$ | This study |
| XLY182 | *rad9:URA3/rad9:URA3* | Derived from strain 3834 |
| XLY184 | *rad9:URA3/rad9:URA3 mcm4*$^{Chaos3/Chaos3}$ | from Judith Berman lab |
| XLY429 | *mad2:URA3/mad2:URA3* | Derived from RHC 15.1 |
| XLY431 | *mad2:URA3/mad2:URA3 mcm4*$^{Chaos3/Chaos3}$ | from Kiwon Song lab |
| XLY270 | *hom3-10/HOM3 can1-100/CAN1* | Derived from MC42-2d |
| XLY462 | *hom3-10/HOM3 can1-100/CAN1* | and HLK1042-1C from |
| | *mcm4*$^{Chaos3/+}$ | Tom Petes lab |
| XLY005 | *hom3-10/HOM3 can1-100/CAN1* | |
| | *mcm4*$^{Chaos3/Chaos3}$ | |
| XLY385 | G1-1 *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY499 | G1-2 *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY386 | G2-1 *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY500 | G2-2 *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY496 | G3 *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY502 | G3P *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY510 | G4 *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY516 | G1-1derivative *mcm4*$^{Chaos3/Chaos3}$ | This study |

**Supplemental Table 1 (Continued)**

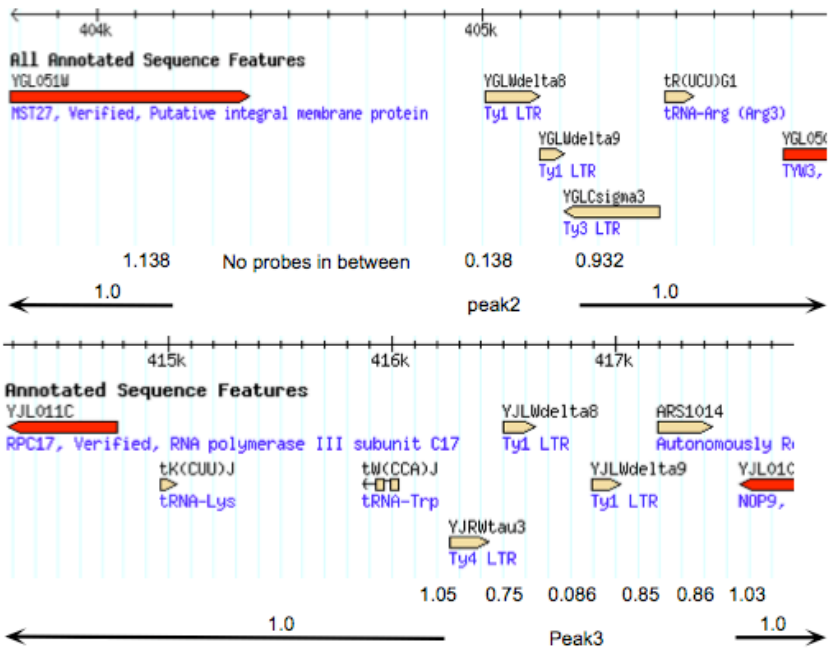
| | | |
|---|---|---|
| XLY536 | G2-1derivative 1 *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY537 | G2-1derivative 2 *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY534 | G2-2derivative *mcm4*$^{Chaos3/Chaos3}$ | This study |
| XLY593 | G1-1-W *mcm4*$^{Chaos3/+}$ | This study |
| XLY82 | *rad51::HIS3/rad51::HIS3* | Derived from strain |
| XLY83 | *rad51::HIS3/rad51::HIS3* | KHKY1039-4D from |
| | *mcm4*$^{Chaos3/Chaos3}$ | Hannah Klein Lab |
| XLY90 | *mrc1::HIS3/mrc1:HIS3* | Derived from strain |
| | *sml1::URA3/sml1::URA3* | YJT134 from John |
| XLY92 | *mrc1::HIS3/mrc1:HIS3* | Diffley Lab |
| | *sml1::URA3/sml1::URA3 mcm4*$^{Chaos3/Chaos3}$ | |
| XLY425 | *tof1::URA3/tof1::URA3* | Derived from strain |
| XLY427 | *tof1::URA3/tof1::URA3 mcm4*$^{Chaos3/Chaos3}$ | YHG3 from Rolf |
| | | Sternglanz Lab |




Supplementary Figure 1. The mouse *Chaos3* mutation F345I is located in a conserved region of MCM4 at the interface between subunits. A) Alignment of amino acid sequence of *MCM4* between different eukaryotes and *Methanobacterium thermoautotrophicum* (mtMCM). The F345I mutation is boxed in red. B) F345 of mouse MCM4 corresponds to F171 of mtMCM. The arrow indicates the location of F171 mtMCM(Fletcher et al. 2003). C) Environment within 5Å of F171 based on the structure of mtMCM(Guex and Peitsch 1997). Different colors represent adjacent subunits of the MCM complex.

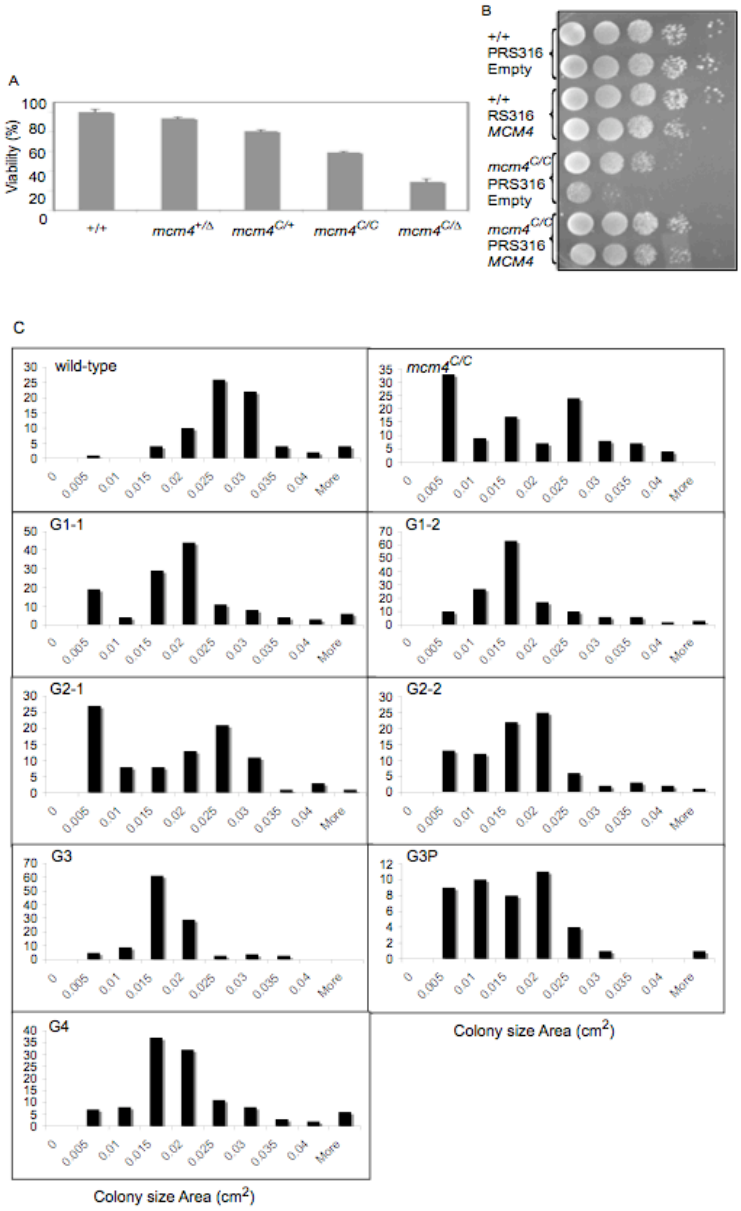



Supplementary Figure 2. Characterization of *mcm4*$^{Chaos3}$ diploid strains. A) Serial dilutions of *mcm4*$^{Chaos3}$ homozgyotes and hemizygotes grown on YPD at 30°C and 37°C. B) Homozygous *mcm4*$^{Chaos3}$ mutants have a higher mitotic index. Log phase cells were analyzed by microscopy. Cells with no bud (G1), small bud (S), and large bud (G2/M) were counted. C) Microscopy of log phase *mcm4*$^{Chaos/Chaos3}$ and wild type cells. D) Fluorescence microscopy of DAPI stained mutant cells. 77% of mutant large budded cells have one nucleus at the bud neck (pointed with white arrow) while 90% of large budded wild type cells have two nuclei.

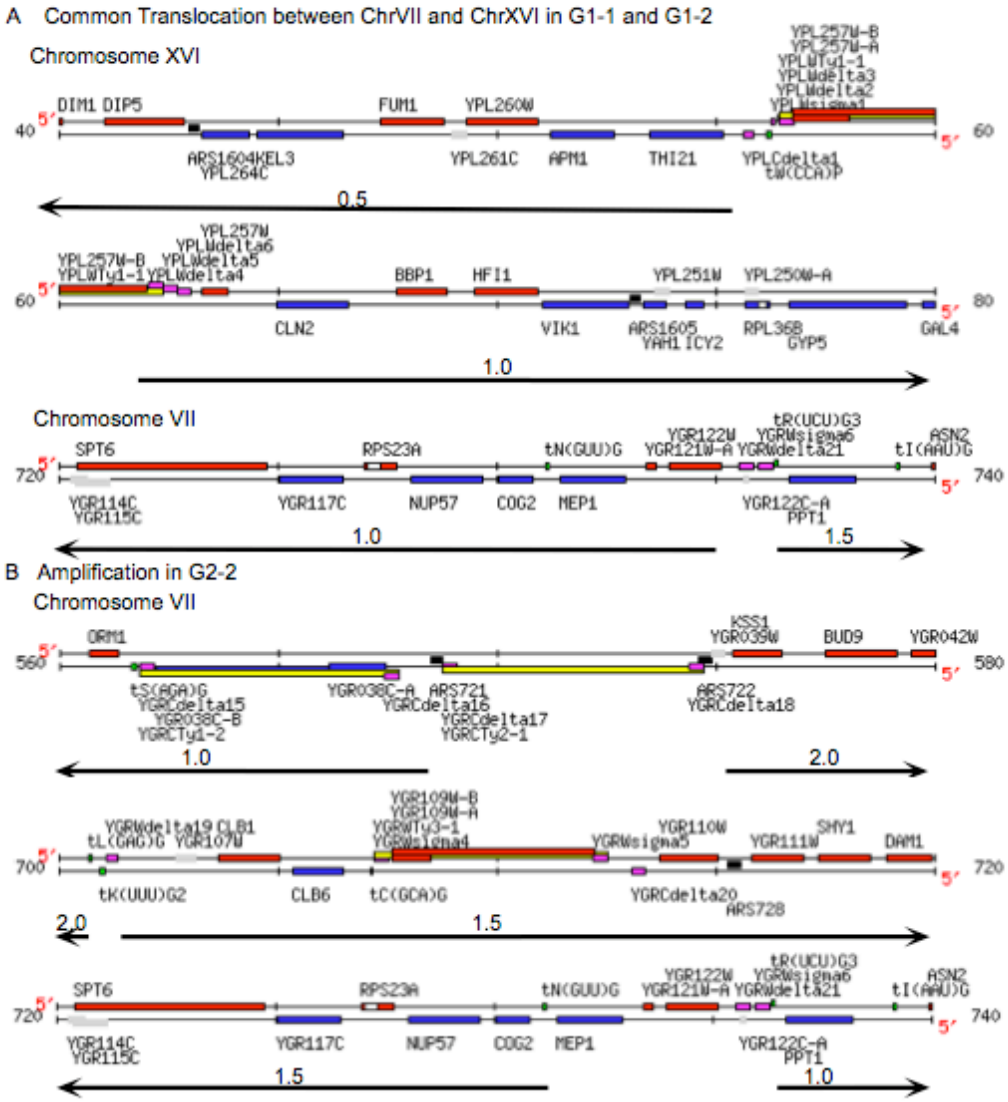


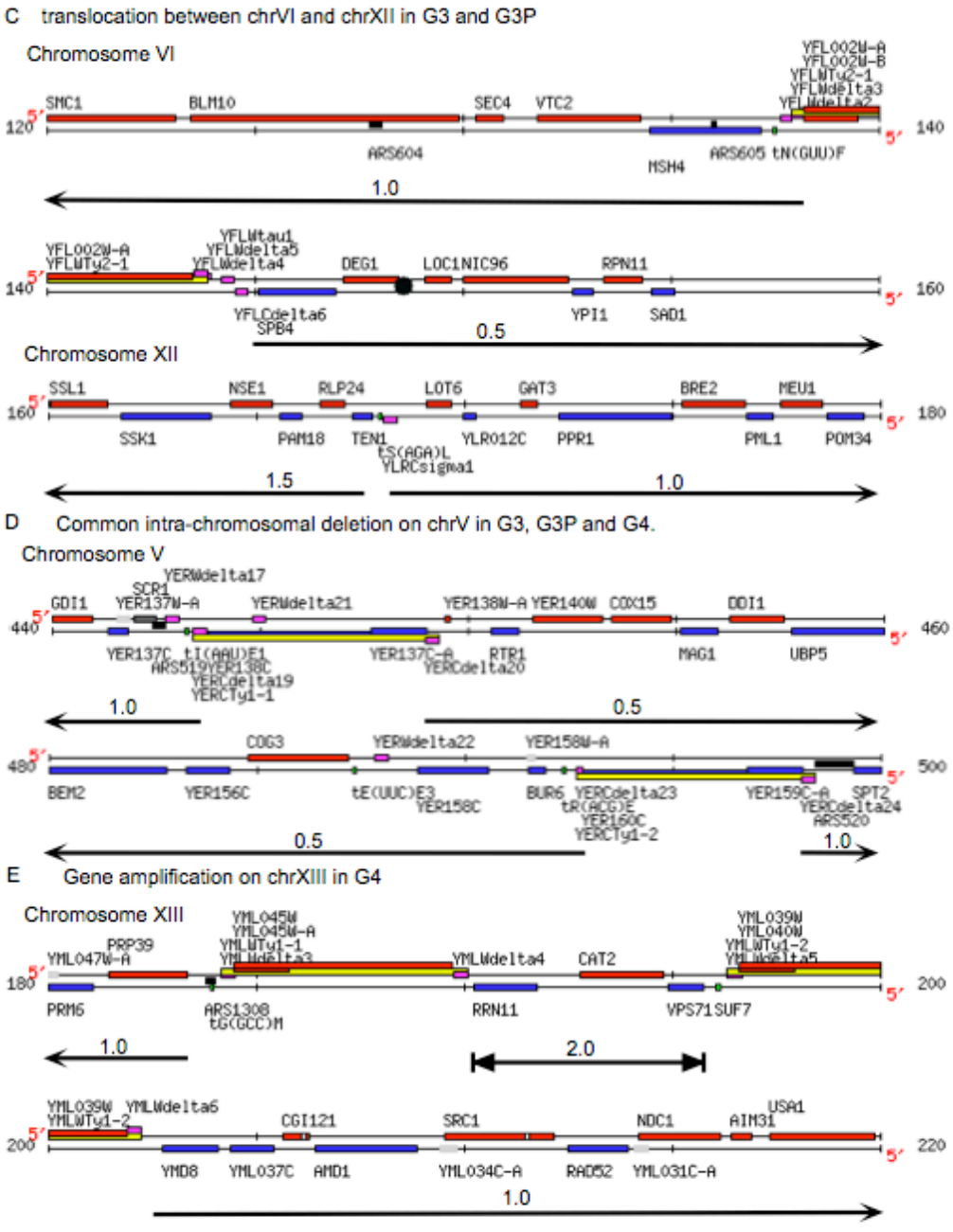

Supplementary Figure 3. Annotated sequence features of the unreplicated regions 2 and 3 shown in Figure 3E. The numbers above the arrows indicate the average DNA amount along the direction of the arrows. The exact DNA copy number of the probes within unreplicated regions are shown in the corresponding locations (Cherry et al. 1997; Harbison et al. 2004; Tachibana et al. 2005).

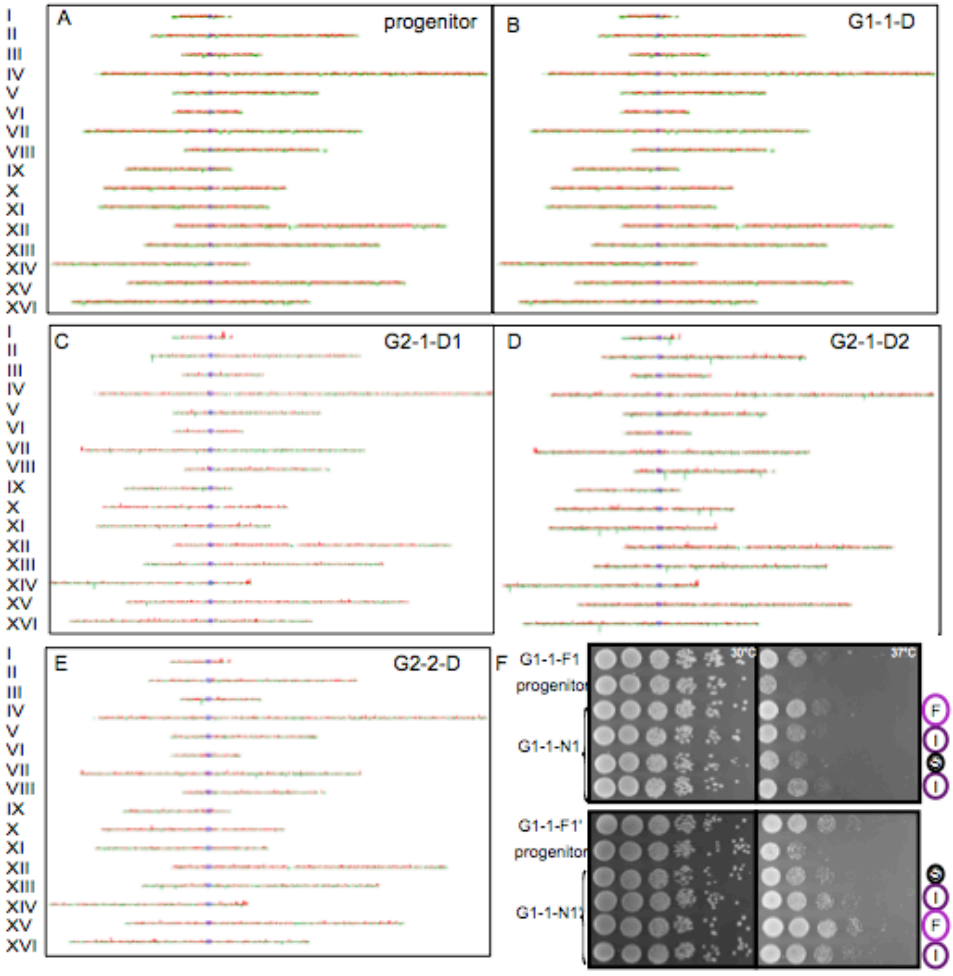
Supplementary Figure 4. A) Cells were grown to log phase, then counted and spread on YPD plates. Viability was measured by counting colonies after 2 days. B) Growth defect of *mcm4*$^{Chaos3}$ diploid is partially rescued by a wildtype *MCM4* on pRS316 at 37°C with empty plasmid as control. Two colonies were picked from each transformants. For *mcm4*$^{Chaos/Chaos3}$ with empty plasmid, one large colony and one small colony are picked. C) Histograms of colony size of wildtype (normal distribution), *mcm4*$^{Chaos3/Chaos3}$ (Bimodal distribution), and fast proliferation strains including G1-1, G1-2, G2-1, G2-2, G3, G3P, and G4 which show varying distributions. The colony sizes were quantified by ImageJ(Rasband 2007).


A
Viability (%)
100
80
60
40
20
0
+/+
mcm4+/Δ
mcm4C/+
mcm4C/C
mcm4C/Δ
B
+/+
PRS316
Empty
+/+
RS316
MCM4
mcm4C/C
PRS316
Empty
mcm4C/C
PRS316
MCM4
C
wild-type
mcm4C/C
G1-1
G1-2
G2-1
G2-2
G3
G3P
G4
0
0.005
0.01
0.015
0.02
0.025
0.03
0.035
0.04
More
Colony size Area (cm2)
Colony size Area (cm2)

Supplementary Figure 5. Chromosomal Features around the breakpoints of genetic aberrations shown in Figure 2. Breakpoints are indicated by the abrupt changes in the DNA copy number along the chromosomes. The numbers above the arrows are the averages of the CGH signals in the direction of the arrows. A) a common translocation between chr VII and XVI in G1-1 and G1-2. This translocation has been confirmed by PCR analysis (data not shown). B) amplification of a 200 kb segment on chrVII that may have involved multiple events in G2-2. C) a common translocation between chr VI and XII in G3 and G3P. D) a common intra-chromosomal deletion on chr V in G3, G3P, and G4. E) amplification of about 5 kb containing *RRN11*, *CAT2*, and *VRS71* on Chr XIII in G4. *Saccharomyces* Genome Database (http://www.yeastgenome.org) (Cherry et al. 1997; Harbison et al. 2004; Tachibana et al. 2005). Red, Watson ORF; blue, Crick ORF; yellow, Ty; pink, LTR.


A Common Translocation between ChrVII and ChrXVI in G1-1 and G1-2
Chromosome XVI
DIM1 DIP5
FUM1
YPL260W
YPL257W-B
YPL257W-A
YPLWTy1-1
YPLWdelta3
YPLWdelta2
YPLWsigma1
ARS1604KEL3
YPL264C
YPL261C
APM1
THI21
YPLCdelta1
tW(CCA)P
0.5
YPL257W
YPLWdelta6
YPL257W-B
YPLWdelta5
YPLWTy1-1
YPLWdelta4
BBP1
HFI1
YPL251W
YPL250W-A
CLN2
VIK1
ARS1605
YAM1
ICY2
RPL36B
GYP5
GAL4
1.0
Chromosome VII
SPT6
RPS23A
tN(GUU)G
YGR122W
YGR121W-A
tR(UCU)G3
YGRWsigma6
YGRWdelta21
ASN2
tI(AAU)G
YGR114C
YGR115C
YGR117C
NUP57
COG2
MEP1
YGR122C-A
PPT1
1.0
1.5
B Amplification in G2-2
Chromosome VII
ORM1
KSS1
YGR039W
BUD9
YGR042W
tS(AGA)G
YGRCdelta15
YGR038C-B
YGRCTy1-2
YGR038C-A
ARS721
YGRCdelta16
YGRCdelta17
YGRCTy2-1
ARS722
YGRCdelta18
1.0
2.0
YGRWdelta19
CLB1
tL(GAG)G
YGR107W
YGR109W-B
YGR109W-A
YGRWTy3-1
YGRWsigma4
YGR110W
YGRWsigma5
SHY1
YGR111W
DAM1
tK(UUU)G2
CLB6
tC(GCA)G
YGRCdelta20
ARS728
2.0
1.5
SPT6
RPS23A
tN(GUU)G
YGR122W
YGR121W-A
tR(UCU)G3
YGRWsigma6
YGRWdelta21
ASN2
tI(AAU)G
YGR114C
YGR115C
YGR117C
NUP57
COG2
MEP1
YGR122C-A
PPT1
1.5
1.0


C translocation between chrVI and chrXII in G3 and G3P
Chromosome VI
SMC1
BLM10
SEC4
VTC2
YFL002W-A
YFL002W-B
YFLWTy2-1
YFLWdelta3
YFLWdelta2
120
140
ARS604
MSH4
ARS605 tN(GUU)F
1.0
YFL002W-A
YFLWTy2-1
YFLWtau1
YFLWdelta5
YFLWdelta4
DEG1
LOC1NIC96
RPN11
140
160
YFLCdelta6
SPB4
YPI1
SAD1
0.5
Chromosome XII
SSL1
NSE1
RLP24
LOT6
GAT3
BRE2
MEU1
160
180
SSK1
PAM18
TEN1
tS(AGA)L
YLRCsigma1
YLR012C
PPR1
PML1
POM34
1.5
1.0
D Common intra-chromosomal deletion on chrV in G3, G3P and G4.
Chromosome V
YERWdelta17
SCR1
GDI1
YER137W-A
YERWdelta21
YER138W-A
YER140W
COX15
DDI1
440
460
YER137C
tI(AAU)E1
ARS519YER138C
YERCdelta19
YERCTy1-1
YER137C-A
YERCdelta20
RTR1
MAG1
UBP5
1.0
0.5
COG3
YERWdelta22
YER158W-A
480
500
BEM2
YER156C
tE(UUC)E3
YER158C
BUR6
YERCdelta23
tR(ACG)E
YER160C
YERCTy1-2
YER159C-A
SPT2
YERCdelta24
ARS520
0.5
1.0
E Gene amplification on chrXIII in G4
Chromosome XIII
PRP39
YML047W-A
YML045W
YML045W-A
YMLWTy1-1
YMLWdelta3
YMLWdelta4
CAT2
YML039W
YML040W
YMLWTy1-2
YMLWdelta5
180
200
PRM6
ARS1308
tG(GCC)M
RRN11
VPS71SUF7
1.0
2.0
YML039W
YMLWTy1-2
YMLWdelta6
CGI121
SRC1
NDC1
AIM31
USA1
200
220
YMD8
YML037C
AMD1
YML034C-A
RAD52
YML031C-A
1.0




Supplementary Figure 6. Accelerated proliferating strains derived by outcrossing the chromosome aberrations. A), B), C), D), E) CGH Analysis of G1-1-D, G2-1-D-1, G2-1-D-2. G2-2-D. F) Tetrads of G1-1-F1 and G1-1-F1' were further backcrossed to the progenitor *mcm4*$^{Chaos3}$ strain. Growth rates of the resulting diploids G1-1-N1 and G1-1-N1' at 30°C and 37°C were compared by plating on YPD plate. All showed a 1:2:1 segregation for the AP phenotype where F = fast, I = intermediate, S = slow.

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CHAPTER 3

# Ploidy Regulation of Fragile Sites Affects Genetic Instability and Repair Pathway Choice under DNA Replication Stress in Yeast


## Abstract

*Mcm4*$^{Chaos3}$, a missense allele, was previously reported to induce adenocarcinomas exclusively in the mammary tissue in >80% of homozygous female mice. Diploid yeast homozygous for an equivalent mutation exhibits severe genetic instability (GIN). However, GIN is not observed in *mcm4*$^{Chaos3}$ haploids. This study dissects the underlying causes for the dichotomy of celluar response to the replication stress presented by a defective MCM helicase in haploids and diploids and underscores the importance of using diploid yeast as a model system. I found that DNA replication stress is induced in both haploid and diploid mutant, but different repair pathways are adopted to counter the DNA damage induced in each cell type. Haploid mutants use the MGS1- and RAD6-dependent pathways that resume stalled forks, whereas the diploid mutants use the RAD52- and MRX- dependent pathways that repair double strand breaks. The diploid specific GIN and repair pathway choice is not determined by mating type heterozygosity or the availability of repair enzymes but by the ploidy. Only in diplophase are the long terminal repeat (LTR) regions vulnerable to replication stress induced by *mcm4*$^{Chaos3}$, which follows tightly coupled error prone recombination at these interspersed repeat sequences. In summary, the DNA replication stress created by the *mcm4*$^{Chaos3}$ mutation results in GIN only in particular cell types. The dichotomous consequences of DNA replication stress stems from the cell-type regulation of the fragility of the interspersed repeat sequences,

which might provide insight into the etiology of tissue specific tumors caused by *Mcm4*$^{Chaos3}$ in mammals

## Introduction

Among the genetic and epigenetic changes of genome, changes in ploidy are among the most drastic such that polyploidy is not tolerated by most animal species (Li et al. 2008). A recent study of tetraploid yeast suggests that the molecular basis of the deleterious effect of ploidy change is due to the uncoordinate scaling of the spindle pole body, spindle and kinetochores, thus leading to genetic instability (GIN) (Storchova et al. 2006). However, ploidy change occurs in every sexual cycle of all eukaryotes. Little is known about how cells deal with the transition from haplophase to diplophase--especially how the DNA damage response is regulated.

DNA replication stress is one of the sources of GIN, and GIN is believed to accelerate tumorigenesis by generating more mutations. Fragile sites are regions of genome that are particularly prone to breaks following partial inhibition of DNA synthesis. These sites are frequently rearranged or deleted in tumor cells (Durkin and Glover 2007; Freudenreich 2007; Smith et al. 2007). Replication forks are more likely to stall or collapse at vulnerable regions of the genome, but the reason for the fragility is still unclear (Durkin and Glover 2007; Lukusa and Fryns 2008). In yeast Ty elements and LTRs are hotspots for translocation under replication stress (Dunham et al. 2002; Lemoine et al. 2005; Admire et al. 2006; Argueso et al. 2008; Lemoine et al. 2008), indicating that both yeast and mammalian genomes exhibit replication-stress-sensitive loci. The Ty and LTR elements may be functionally analogous to mammalian fragile sites and provide a potential model to understand the mechanisms of the fragility and the chromosomal rearrangement that follow.

Animal carriers of the *Mcm4*$^{Chaos3}$ allele, which encodes a defective replication helicase, develop cancer within one year (Shima et al. 2007b). The tumor displays striking tissue specificity. Homozygous females are highly prone to mammary tumors, while males and non-mammary tissues in females are grossly unaffected (Shima et al. 2007a). Mice with reduced MCM2 level mostly develop lymphomas (Pruitt et al. 2007), again demonstrating the tissue specificity of phenotypes associated with *mcm* mutations. Given that DNA replication is ubiquitous to all tissues, it has been a challenge to elucidate the reason for the tumorgenesis as a consequence of DNA replication stress in some but not all cell types.

Cell-type preference for different repair pathways could be one of the explanations for these tissue-specific responses. Double strand breaks (DSBs) are repaired by two main pathways, nonhomologous end-joining (NHEJ) and homologous recombination (HR). Yeast mainly uses the HR pathway. In diploid yeast, NHEJ is severely disabled through the repression of *NEJ1,* a key component of NHEJ, by transcription factor a1-α2. The a1-α2 is coded by the *MAT*a and *MAT* α gene products in diploids (Frank-Vaillant and Marcand 2001). While human somatic cells use NHEJ as the main pathway to repair DSBs (Mao et al. 2008). Mouse embryonic stem cells display enhanced HR capacity, perhaps due to the inactivation of P53 in ES cells (Shrivastav et al. 2008b). However, little is known about the cell type specific regulation for damage repair other than DSBs (Barbour and Xiao 2006; Shrivastav et al. 2008a).

*Saccharomyces cerevisiae* is an excellent model for studying the mechanisms and pathways leading to GIN, and it has also been used as a model for cell type specific regulation. Yeast naturally has three cell types: haploids with two mating types, *MAT*a and *MAT*α, and *MAT*a/α diploids. They have different properties, most of

which can be attributed to different genotypes at the mating type locus, some of which are due to ploidy (Galitski et al. 1999a). Previously I used a diploid yeast strain bearing the cancer susceptibility allele, *mcm4*$^{Chaos3}$, as a model to study the mechanism of replication-stress-induced GIN (Li et al. Submitted). I began our study on *mcm4*$^{Chaos3}$ allele initially in haploid yeast, which are grossly normal (Shima et al. 2007a). Surprisingly, I discovered that the GIN is a diploid-specific outcome. To our knowledge, *mcm4*$^{Chaos3}$ is the only mutation reported that confers a more severe defect in diplophase than haplophase. Clearly, important diploid specific phenotypes would be missed if haploid mutants were used exclusively in yeast genetic studies.

Previously, I showed that GIN in diploid mutants originated from the defective replication of long terminal repeat (LTR) elements, coupled with error prone repair (Li et al. Submitted). The *mcm4*$^{Chaos3}$ haploid not only has no growth defect but also does not show obvious GIN, although both haploid and diploid mutants show evidence of fork defects. I demonstrated that the LTRs are not vulnerable to replication stress in haploid yeast and *mcm4*$^{Chaos3}$ haploids use other repair pathways without generating GIN. The fragility of LTR regions is neither due to the availability of different repair enzymes nor *MAT* locus heterozygosity, but ploidy itself. This study reveals a fundamental difference between haplophase and diplophase on maintenance of their genome integrity, and also has provided a model to study the dichotomous outcome of a ubiquitous replication stress and the choice of repair pathways among different cell types.

## Results

### Unusual ploidy effect: haploid *mcm4*$^{Chaos3}$ mutants are grossly normal without obvious GIN

I showed in Chapter 2, *mcm4*$^{Chaos3}$ homozygotes and *mcm4*$^{Chaos3/\Delta}$ hemizygotes display a G2/M delay prior to anaphase. The G2/M delay depends on the DNA damage checkpoint gene *RAD9* (Li et al. Submitted). However, in *mcm4*$^{Chaos3}$ haploids there is no cell cycle delay observed (Figure1A). The haploid mutant is indistinguishable from wild-type with respect to doubling time (2.00±0.03 vs. 2.02±0.01 hrs).

Since the diploid mutants show a severe GIN with 100-fold increase in loss of heterozygosity (LOH) that is mainly due to hyper-recombination, I investigated the recombination rate in the *mcm4*$^{Chaos3}$ haploid strain with a recombination reporter for intrachromosomal gene conversion and for deletions between direct repeats, which results primarily from single-strand annealing (Xu et al. 2004). The *mcm4*$^{Chaos3}$ mutant has wild-type levels of deletion events ($6.18 \pm 1.96 \times 10^{-5}$ vs $8.57 \pm 1.95 \times 10^{-5}$), and a gene conversion rate ($1.93 \pm 0.48 \times 10^{-5}$) that is slightly higher than that of the wild-type ($1.11 \pm 0.19 \times 10^{-5}$). Thus, unlike the diploid mutant, which exhibits a 100-fold increase in mitotic recombination, the haploid *mcm4*$^{Chaos3}$ mutant does not display hyper-recombination.

I next examined the mutation rate of *mcm4*$^{Chaos3}$ haploid using the *CAN1* forward mutation assay (Kokoska et al. 2000). Haploid *mcm4*$^{Chaos3}$ only showed a subtle mutator phenotype, with a mutation rate ($1.1 \pm 0.2 \times 10^{-6}$) about 2.5-fold above wild-type ($3.9 \pm 0.1 \times 10^{-7}$). The slight increase of the mutation rate in the *mcm4*$^{Chaos3}$ haploids prompted us to examine the potential increase of gross chromosome rearrangement (GCR) frequency in the ChrXV-L GCR strain (Kanellis et al. 2007), in

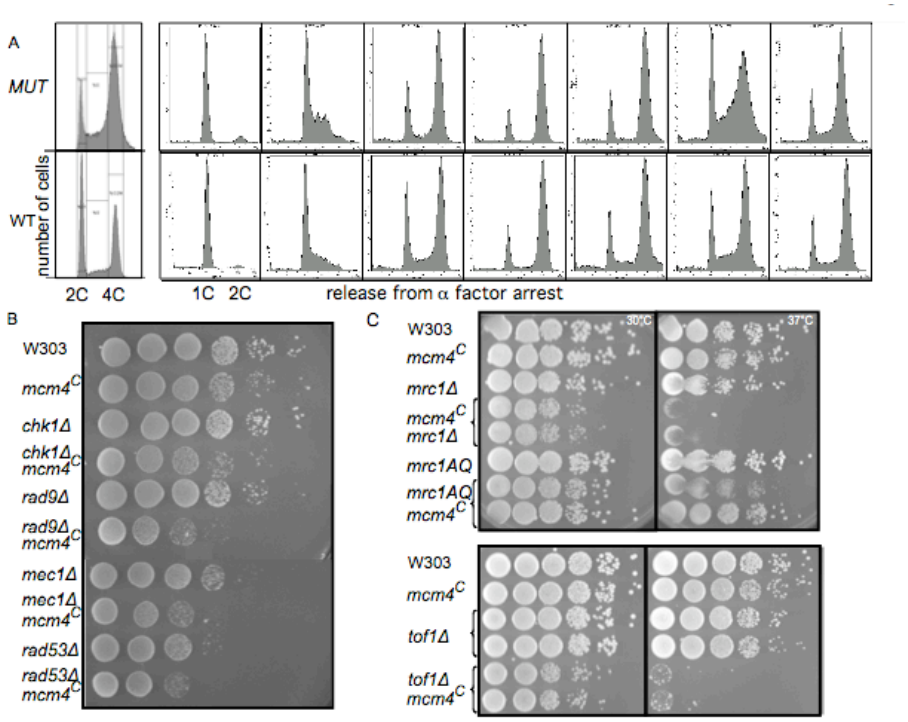



Figure 1. The normal growth of *mcm4*$^{Chaos3}$ haploid requires intact checkpoint functions and fork stabilization proteins. A) The haploid mutant and wild type are arrested with α factor and release to fresh medium, and cell cycle progression is monitored by FACS every 20 minutes. The cell cycle distribution of log phase diploid mutant and wild-type are listed on the right. B) *mcm4*$^{Chaos3}$ shows synthetic growth defects with checkpoint mutants. Tenfold serial dilutions of each strain were spotted on complete medium plates and incubated at 37°C. C) *mcm4*$^{Chaos3}$ shows synthetic lethality with fork stabilizing mutations *mrc1Δ* and *tof1Δ* at 37°C, and the synthetic lethality is not seen in the *mrc1AQ* mutant.

which the loss of two selectable markers, the *CAN1* and *URA3* genes, ~10 kb from the telomere of ChrXV-L, was measured. I observed no dramatic increase of GCR in the mutant strain ($10.0 \pm 0.9 \times 10^{-8}$) in comparison to wild-type ($6.0 \pm 1.0 \times 10^{-8}$).

In summary, *mcm4*$^{Chaos3}$ causes a cell cycle delay and severe GIN in diploid yeast. However, these phenotypes were not observed in *mcm4*$^{Chaos}$ haploids. These disparate observations suggest an unusual dependence on ploidy for the manifestation of GIN stimulated by the *mcm4*$^{Chaos3}$ allele.

**_mcm4_$^{Chaos3}$ haploid requires intact checkpoint functions and fork stabilization proteins for normal growth**

There are two explanations for the subtle GIN phenotype and the absence of a G2/M delay in the haploid mutant. First, there is no, or a very small amount of DNA damage incurred in *mcm4*$^{Chaos3}$ haploids. Second, the repair pathway in haploid is robust and efficient enough to deal with the damage induced by *mcm4*$^{Chaos3}$ without generating GIN and delaying the cell cycle. To distinguish between these two possibilities, double mutants of *mcm4*$^{Chaos3}$ were constructed with various checkpoint mutations. If *mcm4*$^{Chaos3}$ caused only subtle damage in haploids, the checkpoints would not be activated, and checkpoint response pathways would be dispensable. Thus, these double mutant strains of *mcm4*$^{Chaos3}$ and checkpoint mutations would be normal. Conversely, if *mcm4*$^{Chaos3}$ causes a great amount of DNA damage in haploids, cells with checkpoint mutations could not detect the damage generated by *mcm4*$^{Chaos3}$, which will lead to unrepaired damage and severe growth defects. Supporting the second possibility, *mcm4*$^{Chaos3}$ showed synthetic growth defect with all tested checkpoint mutants, including *chk1Δ*, *rad9Δ*, *mec1Δ*, and *rad53Δ* (Fig 1B). These results indicate that *mcm4*$^{Chaos3}$ also induced DNA damage in haplophase.

The homozygous diploid *mcm4*$^{Chaos3}$ was defective in DNA replication and showed synergistic growth defects with *mrc1Δ* and *tof1Δ*. Mrc1 and Tof1 are replication fork stabilization proteins that are loaded onto DNA shortly after initiation and travel with the replication fork (Katou et al. 2003). To investigate whether DNA damage in the haploid also originated from replication forks, double mutants of *mcm4*$^{Chaos3}$ with fork stabilization mutations were constructed in the haploid. I found that the double mutant of *mcm4*$^{Chaos3}$ with *mrc1Δ* or *tof1Δ* is synthetically lethal at the restricted temperature in haplophase (Figure 1C), suggesting that damage in haploid was also generated at the forks. This synthetic effect in haplophase is more severe than in diplophase. To verify that this synergistic effect is the result of an enhanced destabilization of the replication fork rather than an exacerbated deficiency in replication checkpoint response, I examined the genetic interaction between *mcm4*$^{Chaos3}$ and *mrc1-AQ*. The mrc1-AQp is deficient in the replication checkpoint response but its association with the replication fork is unaffected (Osborn and Elledge 2003). There was no severe synergy observed in the double mutant of *mrc1-AQ* and *mcm4*$^{Chaos3}$, suggesting that the essential function of Mrc1p in *mcm4*$^{Chaos3}$ is mostly fork stabilization (Figure 1C).

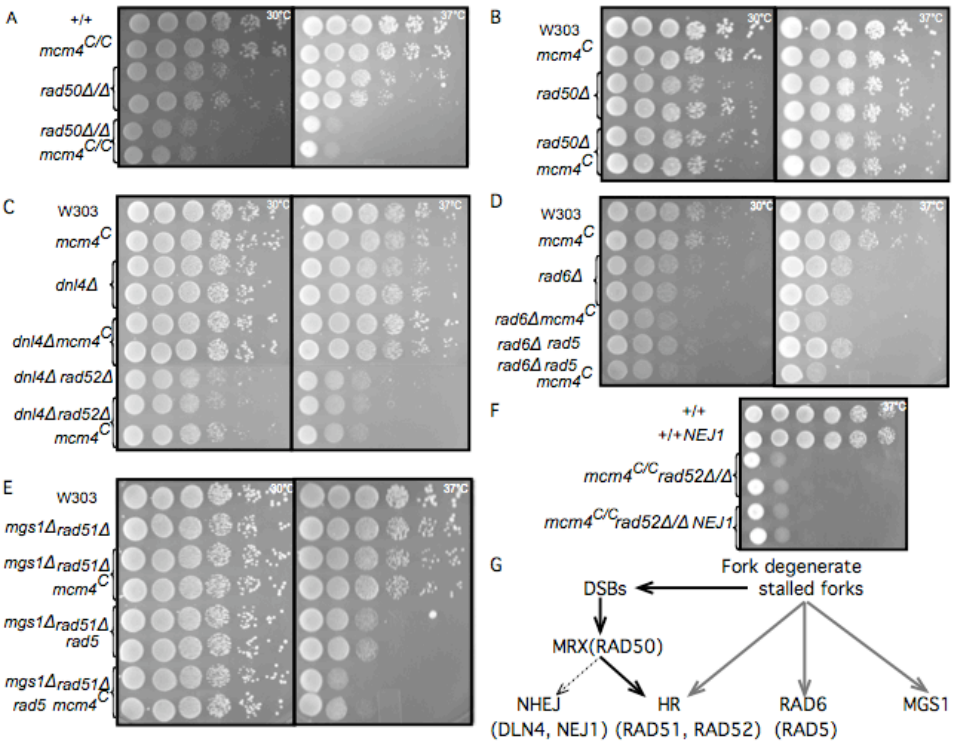
Our results indicate that both haploid and diploid mutants suffer from fork defects and require the cooperation of fork stabilization proteins for survival. Previously I showed that DNA replication defects coupled with error prone repair are the underlying mechanisms of GIN induced by *mcm4*$^{Chaos3}$. If haploids and diploids both experience similar fork defects, then the diploid specific GIN and growth defects must be caused by distinctive repair mechanisms downstream of the fork damages with haploid cells being able to repair the fork damage more efficiently without causing GIN.

**Diploid mutant requires the DSB repair pathway, while haploid mutant does not**

I have shown that the Rad51 dependent HR is essential in *mcm4*$^{Chaos3}$ diploids. There are three principal lesions that could trigger spontaneous HR: DSBs, stalled replication forks and collapsed forks (Saleh-Gohari et al. 2005). DSB recognition and kinase activation of ATM/Tel1p are mediated through the Mre11-Rad50-Xrs2 (MRX) protein complex (Costanzo et al. 2004). An increase in DSBs should result in a greater requirement for MRX function. Consistent with this idea, I found *mcm4*$^{Chaos3}$ and *rad50Δ* were synthetically lethal at 37 °C in diplophase (Fig 2A). Therefore, the DSBs repair pathway (DSBR) is indispensable for *mcm4*$^{Chaos3}$ diploids.

Other than HR, another independent DSB repair pathway is NHEJ, which is sequestered in diploids (Frank-Vaillant and Marcand 2001). It is likely that DSBs are also generated in the *mcm4*$^{Chaos3}$ haploid, and NHEJ is the more efficient pathway preventing DSBs from translating into GIN. To test this hypothesis, I constructed double and triple mutants of *mcm4*$^{Chaos3}$ with DSB repair mutants in haploid. Dnl4 (DNA ligase IV) is a key component of NHEJ pathway (Martin et al. 1999). Double mutant of *mcm4*$^{Chaos3}$ with *dnl4Δ* or *rad50Δ* grew as well as wild-type in haplophase (Fig 2B and 2C). Disruption of both the HR and the NHEJ pathways did not show synergistic defects with *mcm4*$^{Chaos3}$ in the triple mutant (Figure 2C). Thus the haploid mutant does not require DSBR, suggesting the haploid mutant does not experience DSBs as diploid mutant does, but certain damage other than DSBs.

Figure 2. Distinct repair pathways are used in haploid and diploid mutants. A) The diploid *mcm4*$^{Chaos3/Chaso3}$ *rad50Δ/Δ* double mutant shows synthetic lethality at 37°. B) The haploid *mcm4*$^{Chaos3}$ *rad50Δ* double mutant is grossly normal. C) The haploid *mcm4*$^{Chaos3}$ *dnl4Δ* double mutant is grossly normal. The *mcm4*$^{Chaos3}$ does not show further synthetic effect with *dnl4Δ* or *rad52Δ*. D) The haploid *mcm4*$^{Chaos3}$ *rad6Δ* double mutant shows synthetic growth defect, and this defect is not accelerated with *rad5* mutant. E) The haploid quadruple mutant of *mcm4*$^{Chaos3}$ *mgs1Δ rad52Δ rad5* is very sick compared to the triple mutant of *mgs1Δ rad52Δ rad5*. F) The diploid *mcm4*$^{Chaos3/Chaso3}$ *rad52Δ/Δ* double mutant shows synthetic lethality at 37°, and this lethality cannot be rescued by ectopic expression of *NEJ1* with empty vector as control. G) Summary picture of the repair pathway used in haploid and diploid mutants. Black arrows indicate the pathway used in haploid, while grey arrows indicate the diploid pathway. The dash arrow indicates a potential but unused pathway.


A
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mgs1Δrad51Δ rad5
mgs1Δrad51Δ rad5 mcm4C
F
+/+
+/+NEJ1
mcm4C/Crad52Δ/Δ
mcm4C/Crad52Δ/Δ NEJ1
G
Fork degenerate stalled forks
DSBs
MRX(RAD50)
NHEJ (DLN4, NEJ1)
HR (RAD51, RAD52)
RAD6 (RAD5)
MGS1

**Haploid mutant requires the *RAD6* and *MGS1* dependent stalled fork resumption pathway**

To dissect the repair pathways in $mcm4^{Chaos3}$ haploids, I refer to Chapter 2 that diploid mutant has defective replication at the LTR regions (Li et al. Submitted). I suspected that stalled forks are the primary damage caused by $mcm4^{Chaos3}$. Other than HR, cells can resume replication at stalled forks via a *RAD6*-dependent pathway and a novel *MGS1*-dependent pathway (Leslie Barbour, 2003). I constructed double, triple, and quadruple mutants of $mcm4^{Chaos3}$ with those mutations defective in repairing stalled forks. I found $mcm4^{Chaos3}$ showed synthetic growth defect with *rad6Δ* (Figure 2D). However, the substantial viability of $mcm4^{Chaos3}$ and *rad6Δ* suggested that other pathways are employed. Severe growth defects in the $mcm4^{Chaos3}$ strain were observed only when I disabled both the HR and *MGS1* dependent pathways as well as partially disable the *RAD6* dependent pathway by a *rad5* point mutation (Fan et al. 1996; Ulrich and Jentsch 2000) (Figure 2E). Unlike the diploid mutant, which relies on the DSBR pathway for survival, the haploid mutant activates multiple pathways to resume the stalled forks, probably before the stalled forks are degenerated to DSBs.

**The damage in diploid mutant cannot be repaired by NHEJ pathway**

I showed in Chapter 2 that the diploid mutant has a fork defect which requires Mrc1 and Tof1 for fork stability (Li et al. Submitted), and the replication stress in haploids seems to be manifested as stalled forks. It is likely that the substrates that activate DSBR in the diploid mutant may not be the conventional DSBs, but may have been derived from collapsed forks. A fork collapse produces a one-ended DSB that has no second end with which to rejoin, and therefore should not be a substrate for NHEJ pathway (Meena Shrivastav, 2008). To test this hypothesis, I activated the NHEJ pathways in diploid by ectopically expressing the *NEJ1* gene, which should

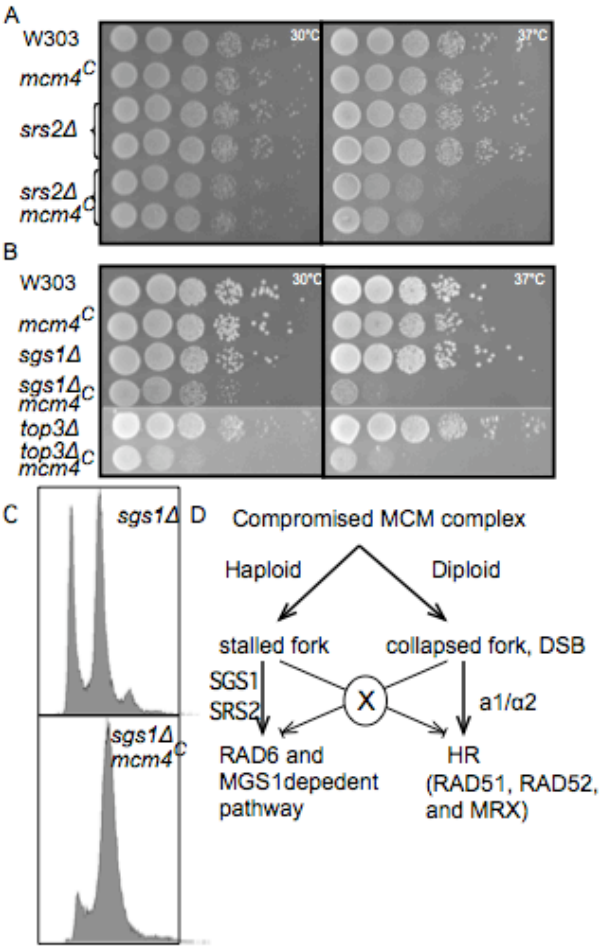
rescue the lethality of the *mcm4*$^{Chaos3}$ *rad52Δ* double mutant if two-ended DSBs were created (Valencia et al. 2001). However, the activation of NHEJ did not repair the damage created in *mcm4*$^{Chaos3}$ diploid (Figure 2E). This result, together with the lower requirement for fork stabilizing proteins in diploid mutants compared to haploids, suggests that the "DSBs" in diploid might be collapsed forks, arising from stalled forks. Thus, stalled forks and collapsed forks may be two outcomes of the replication stress caused by *mcm4*$^{Chaos3}$ that activate different downstream repair pathways (Figure 2G).

In summary, haploid and diploid yeast use distinct repair pathways for the fork defect created by *mcm4*$^{Chaos3}$. I previously showed that HR at interspersed repeat sequences are error prone, which generates GIN in diploid mutant (Li et al. Submitted). Does the choice of repair pathway underlie the diploid specificity of *mcm4*$^{Chaos3}$? What determines the choice of different repair pathways? Is it determined by the availability of repair pathway in an *ad hoc* basis or by an upstream process? I decided to alter the availability of repair pathway in haplophase and diplophase to test if haploid and diploid mutants can be coerced into using either repair pathway to repair their fork defects, and whether the diploid specific growth defect is rescued by channeling into alternative pathways.

**The choice of repair pathway is not reversible and is not determined by the level of repair proteins**

The fork resumption pathway used in the *mcm4*$^{Chaos3}$ haploid is present in diploid (Barbour and Xiao 2006). The fact that the *mcm4*$^{Chaos3}$ is synthetically lethal with *rad52Δ* (Figure 2E) already indicates that the damage generated in diplophase cannot be channeled to other pathways for repair when HR is blocked. Thus, the decision to use the HR pathway is not regulated by the availability of repair pathways,

Figure 3. Damage in haploid mutant cannot be repaired by the pathway used in diploid mutant. A) *mcm4*$^{Chaos3}$ shows synthetic growth defects with *srs2* mutant. B) *mcm4*$^{Chaos3}$ shows synthetic lethality with *sgs1Δ* and *top3Δ* at 37°. C) *sgs1Δ mcm4*$^{Chaos3}$ double mutant is grown to log phase at 30°C and then shifted to 37°C for three hours. FACS profile shows that most cells are arrested with about 2C DNA. D) Summary picture indicates the choice of repair pathway is not reversible, but dictated by the type of DNA damage, which is further regulated by ploidy. The mating type heterozygosity regulates the HR pathway used in diploid as shown in Figure 4.


A
W303
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B
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mcm4C
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top3Δ
top3Δ mcm4C
30°C
37°C
C
sgs1Δ
sgs1Δ mcm4C
D
Compromised MCM complex
Haploid
Diploid
stalled fork
collapsed fork, DSB
SGS1
SRS2
X
a1/α2
RAD6 and MGS1depedent pathway
HR (RAD51, RAD52, and MRX)

and defects in the diploid are not dependent upon the HR pathway. Therefore, distinct repair pathways cannot be the ultimate reason for the diploid specific defects.

Two DNA helicases, SGS1 and SRS2, redundantly regulate the repair pathways that resume stalled forks (Barbour and Xiao 2003). Sgs1 and Srs2 process recombination intermediates formed during fork stalling and channel the damage into the *RAD6*-dependent pathway for repair, and *sgs1Δ* or *srs2Δ* appears to have unrestrained recombination (Gangloff et al. 2000). In haploids, I used *sgs1Δ* and *srs2Δ* to channel damage from the fork resumption pathway to HR, a pathway that is essential in the diploid mutant. The $mcm4^{Chaos3}$ and *srs2Δ* haploid mutant showed synthetic growth defects at the restricted temperature (Figure 3A). The effect of the $mcm4^{Chaos3}$ and *sgs1Δ* double mutant is even more dramatic, showing a synthetic lethality at the restricted temperature (Figure 3B) with cell cycle arrest at late S or G2/M phase (Figure 3C), indicating the DNA damage substrate created by $mcm4^{Chaos3}$ in haploids cannot be repaired by HR. Thus, the choice of repair pathway in haploid is not determined by availability, but is regulated by a process upstream of the recruitment of the SGS1 and SRS2 helicases.

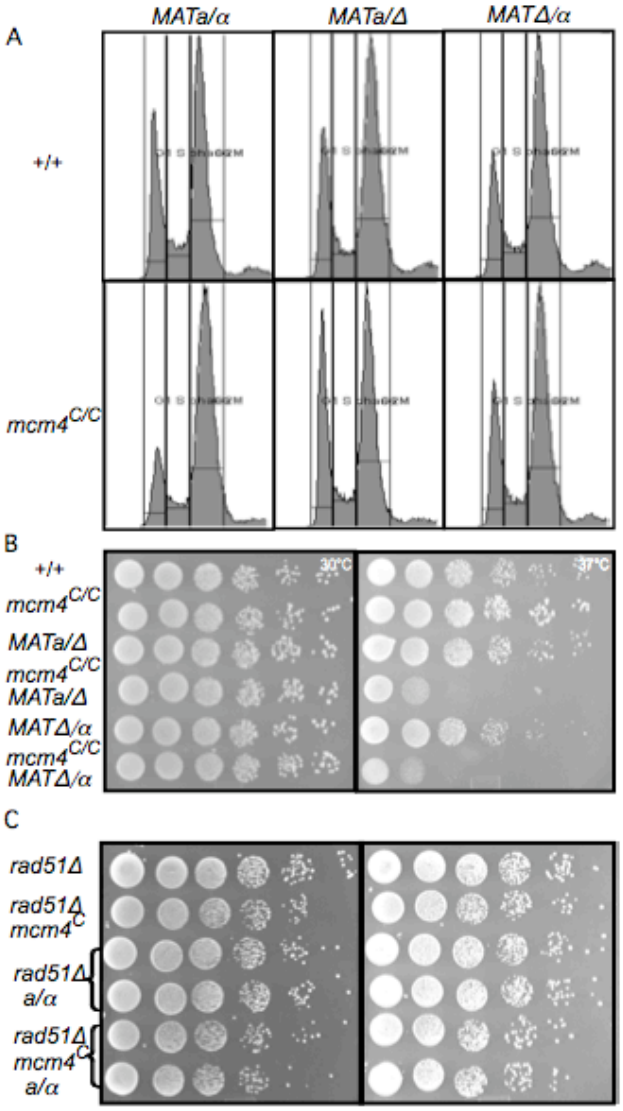
In summary, haploid and diploid mutants are unable to use each other's repair pathways to repair their fork defects (Figure 3D), indicating the choice of repair pathway is probably regulated by different initial processing steps that depend on how the replication defects are generated. What determines this ploidy difference, and why are the diploid more susceptible to the damage caused by $mcm4^{Chaos3}$? The two obvious determinants for cell type identity are mating type heterozygosity and ploidy itself.

**The diploid specific effect is not due to *MAT* locus heterozygosity**

Diploid yeast strains are more resistant than haploid strains to γ-rays, UV and methyl methanesulphonate (MMS). This resistance is in part due to heterozygosity at the *MAT* locus (Heude and Fabre 1993; Barbour and Xiao 2006). The effect of *MAT* heterozygosity on increased resistance to DNA damage agents is dependent on the function of HR proteins (Saeki et al. 1980). To investigate whether the unusual diploid specificity of the $mcm4^{Chaos3}$ phenotype is due to heterozygosity at the *MAT* locus, I constructed the *MATa*/*Δ* and *MATΔ*/*α* diploid. These $mcm4^{Chaos3}$ diploids hemizygous at the *MAT* locus no longer exhibit a G2/M delay (Figure 4A), but are inviable at the restricted temperature (Figure 4B), indicating that the damage can no longer be repaired. The fact that the growth defect in diploid mutants with *MAT* hemizygosity is worse than with *MAT* heterozygosity indicates that the diploid specific growth defects are not due to *MAT* heterozygosity.

To investigate the GIN in $mcm4^{Chaos3}$ diploid mutant with *MAT* homozygosity, I measured the LOH frequency of *CAN1* with respect to *HOM3* on the left arm of chromosome V (Hartwell and Smith 1985). There was little difference in the frequency between *MAT*a/α $mcm4^{Chaos3/Chaos3}$ ($2.60 \pm 1.60 \times 10^{-3}$) and *MATΔ/α* $mcm4^{Chaos3/Chaos3}$ ($1.02 \pm 0.49 \times 10^{-3}$) yeast, which is about 100-fold elevated over that of the wild type ($2.12 \pm 0.11 \times 10^{-5}$). However, almost all LOH events in *MATΔ*/α $mcm4^{Chaos3/Chaos3}$ were also due to mitotic recombination as they are in *MAT*a/*α* $mcm4^{Chaos3/Chaos3}$. This result indicates that the decision of using HR in diploid mutants or diploid specific GIN is independent of *MAT* heterozygosity. The fact that *MAT* heterozygosity is required for viability in $mcm4^{Chaos3}$ diploid indicates its role in enhancing the efficiency of HR pathway, consistent with the previous finding that

Figure 4. Mating type heterozygosity confers the *mcm4*$^{Chaos3}$ diploid checkpoint activation and appropriate repair. A) FACS analysis of a and α *mcm4*$^{Chaos3}$ diploids do not show a G2/M delay compared to a and α wild-type diploids. The a/α *mcm4*$^{Chaos3}$ diploid and wild-type is listed as control on the left. B) *MAT*a/*Δ* and *MATΔ*/α *mcm4*$^{Chaos3}$ diploids are lethal at 37°, while the a and α wild-type diploids are almost normal. C) The a/α *mcm4*$^{Chaos3}$ *rad51Δ* haploid mutant is grossly normal compared to a/α *rad51Δ*, a *mcm4*$^{Chaos3}$ *rad51Δ* and a *rad51Δ* haploid mutant.


A
MATa/α
MATa/Δ
MATΔ/α
+/+
mcm4C/C
B
+/+
mcm4C/C
MATa/Δ
mcm4C/C
MATa/Δ
MATΔ/α
mcm4C/C
MATΔ/α
30°C
37°C
C
rad51Δ
rad51Δ mcm4C
rad51Δ a/α
rad51Δ mcm4C a/α

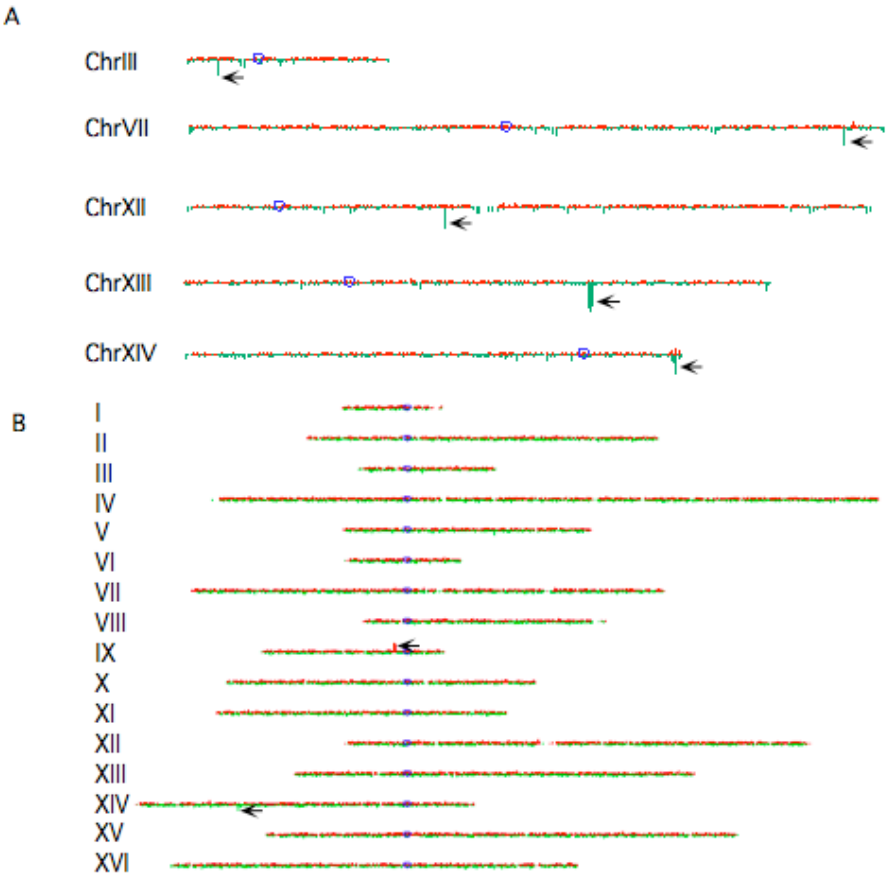
*MAT* heterozygosity alters DNA repair in yeast by several independent targets (Valencia-Burton et al. 2006).

In order to confirm that the diploid specific defect is not due to *MAT* heterozygosity, I constructed the double mutant *mcm4*$^{Chaos3}$ *rad51Δ* in a haploid with *MAT* heterozygosity. The diploid double mutant *mcm4*$^{Chaos3}$ *rad51Δ* was previously shown to be lethal at the restricted temperature (Li et al. Submitted), while the haploid double mutant is grossly normal (Figure 4C). The *MATa/α mcm4*$^{Chaos3}$ *rad51Δ* haploid did not show any synthetic effect (Figure 4C). Therefore, the diploid specific defect and repair pathway choice is due to the ploidy, and the replication defects induced by *mcm4*$^{Chaos3}$ are either generated or processed more detrimentally in diploid than that is in haploid yeast.

**LTR regions are not fragile in *mcm4*$^{Chaos3}$ haploid mutant**

In Chapter 2, I showed that the LTR regions are fragile sites in *mcm4*$^{Chaos3}$ diploid, which are defective for replication and display hot spots for recombination (Li et al. Submitted). In order to check whether haploid mutant also has defective replication at LTRs, I use a similar approach to map the replication barriers in *mcm4*$^{Chaos3}$ haploid by blocking the repair of the region with defective replication. I took advantage of the ts phenotype of *mcm4*$^{Chaos3}$ *sgs1Δ* cells, in which the fork defect in *mcm4*$^{Chaos3}$ is created but cannot be repaired so that the potential fork barrier zones should be under-replicated at the restrictive temperature relative to other regions in the genome. Using CGH, I observed several under-replication regions. Contrary to expectation, none of them were located in LTR regions (Figure 5A). To rule out the influence of *sgs1Δ*, the same strategy was used for the *mcm4*$^{Chaos3}$ *rad52Δ mgs1Δ rad5* strain, in which the under-replicated regions did not localize at specific sites either

Figure 5. Mapping of replication barriers in haploid by CGH analysis. A) and B) mutant is grown to log phase at 30°C and then shifted to 37°C for three hours. A) the CGH analysis of *sgs1Δ mcm4*$^{Chaos3}$ double mutant. Five under-replicated regions with >4-fold decrease in DNA were observed. The peak on Chr XIII corresponds to *Sgs1*, which is deleted in the mutant strain; this serves to validate the array sensitivity. None of the peaks localize to solo LTRs (Supplementary Fig 6). B) the CGH analysis of quadruple mutant of *mcm4*$^{Chaos3}$ *mgs1Δ rad52Δ rad5*. There is no obvious under-replicated region, except the region on ChrXIV corresponding to *Mgs1*, which is deleted in the mutant strain. The peak on ChrIX represents the *bar1* locus, which is deleted in the control strains I use to conduct CGH but not in the mutant strain.


A
ChrIII
ChrVII
ChrXII
ChrXIII
ChrXIV
B
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI

(Fig 5B), suggesting that fork stalling does occur in haploids but not at LTRs. Therefore, the fragility of LTR regions is diploid specific, which probably is the reason for the diploid specific defect and the different repair pathways used in haploid and diploid.

**The fragility of LTR is under ploidy regulation independent of *MAT* heterozygosity**

If the fragility of LTRs is the reason for the diploid specific effect, the *MATa*/*Δ* and *MATΔ/α* diploid mutants should also be defective in replicating the LTR regions. Because the *MATa*/*Δ* and *MATΔ/α* $mcm4^{Chaos3}$ diploids are inviable at the restricted temperature, I used these strains to map the replication barriers at restricted temperature by CGH. I observed that the LTR regions in *MATa*/*Δ* and *MATΔ/α* diploid mutants are over-replicated (Fig 6A and B). These results indicate that LTR regions are fragile in $mcm4^{Chaos3}$ diploids with hemizygous *MAT* locus. The over-replication is probably due to a failure of appropriate HR repair. In summary, the fragility of LTR regions is diploid specific and independent of *MAT* heterozygosity.

**Discussion**

**Choice of repair pathway is under active control rather than passive shunting**

Cells have developed multiple pathways to deal with a particular type of DNA damage. These pathways are distinct regarding repair efficiency and mutagenic potential and must be tightly controlled to preserve viability and genomic stability. It is widely believed that the choice of repair pathways is determined through competition among the pathways for substrate (Gudmundsdottir et al. 2007). In our study, although fork resumption pathway and DSB repair pathways are available in both haploid and diploid, the choice is regulated by the ploidy of the cell type. I showed that the haploid and diploid mutants are unable to use each other's repair

pathways to repair their fork defects (Figure 2F and 3). In *mcm4*$^{Chaos3}$ diploid, *MAT* heterozygosity confers the appropriate HR repair pathway (Figure 4B), but does not determine the choice of going to this repair pathway (Figure 5). Our study suggests that the haploid and diploid repair pathways do not randomly compete for substrates on an *ad hoc* basis, and underscores the importance of the association between ploidy and appropriate DNA repair pathways for genome stability maintenance.

In a particular cell type, the choice of repair pathway seems to be passively shunted among available repair pathways (Gudmundsdottir et al. 2007), however, our results show the contrary. Why did our study reach different conclusions from previous studies? I believe it is due to a direct coupling of repair pathway with fragile sites. In our study, the fork defects at the fragile sites may be processed to collapsed forks as shown in diploid, while, the fork defects outside of fragile sites are rescued directly without collapsing as shown in haploid (Figure 3D). This suggests a complex regulation of repair pathways at fragile sites.

Our study indicates that cells have the capacity to repair replication fork damage without generating GIN. Inappropriate repair can have dire consequences including diseases. As I gain a better understanding of the mechanism that regulates repair pathway choice in different cell types, these basic mechanistic insights will eventually translate into clinical benefits. The model I have developed in yeast is excellent for understanding the mechanism for cell type specific maintenance of genome integrity and for identifying potential gene targets for alleviating the GIN caused by replication stress.

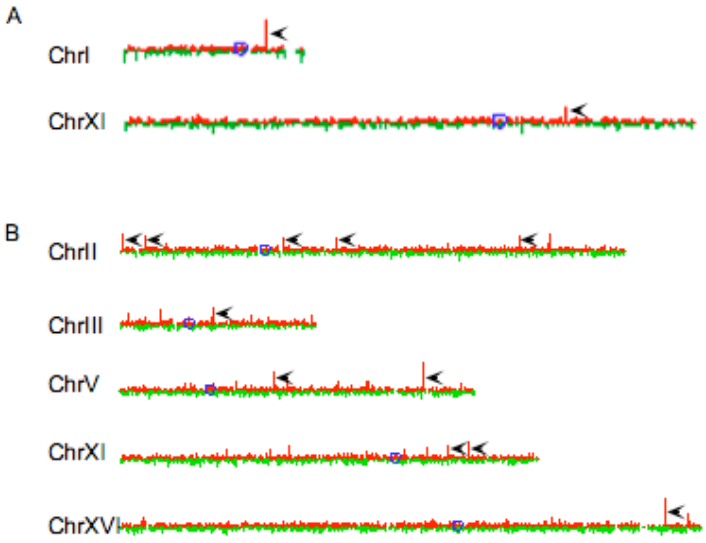



Figure 6. Mapping of replication barriers in *MAT*a/*Δ* and *MATΔ*/α *mcm4*$^{Chaos3}$ diploid by CGH analysis. The *MAT*a/*Δ* and *MATΔ*/α *mcm4*$^{Chaos3}$ diploid mutant is grown to log phase at 30°C and then shifted to 37°C for three hours. A) *MAT*a/*Δ* *mcm4*$^{Chaos3}$ diploid, two peaks over-replicated. B) *MATΔ*/α *mcm4*$^{Chaos3}$ diploid, multiple peaks with over-replication. All the peaks correspond to locations that abut LTRs.

**DNA replication stress does not necessarily cause GIN**

GIN, a hallmark of cancers (Loeb 2001; Rajagopalan et al. 2003), is believed to be the driving force of tumorigenesis. Exploring the origin of GIN is essential to understanding the early events in tumor development. DNA replication stress has been proposed to be a source of GIN (Schar 2001). Using a cross-model organism approach, I showed that it is the DNA replication defect from a defective helicase that causes GIN in both mice and yeast (Li et al. Submitted). However, DNA replication defects do not always lead to GIN. In *Mcm4*$^{Chaos3/Chaos3}$ mice, GIN is only found in the mammary gland of female mice and progenitors of erythrocytes (Shima et al. 2007a), otherwise the mice are grossly normal. What tips the balance causing *Mcm4*$^{Chaos3}$ induced replication defects to favor GIN in female mammary gland, whereas in other tissues towards normality? This complex problem can be addressed by modeling in simple organisms like *Saccharomyces cerevisiae*.

In diploid yeast, I previously showed that *mcm4*$^{Chaos3}$ leads to LOH and a hypermutable subpopulation gains new traits such as aneuploidy and accelerated proliferation (Li et al. Submitted). In contrast, the haploid *mcm4*$^{Chaos3}$ mutant is grossly normal. It is tempting to pin this difference on the fragile sites in the *mcm4*$^{Chaos3}$ mutants. Only in *mcm4*$^{Chaos3}$ diploid, the interspersed repeat regions, LTRs, display their fragility during replication, resulting in DSBs that are repaired by error prone recombination. In contrast, the LTRs are not vulnerable to replication stress in haploid yeast and stalled forks are restored using other repair pathways without generating GIN. I believe that the replication of repetitive DNA is tightly regulated and coordinated to ensure faithful DNA replication and repair in different cell types.

The haploid mutant provides an excellent comparison for characterizing the molecular basis of GIN caused by *mcm4*$^{Chaos3}$ in diploids. Using haploid mutant as a

reference, I am able to confirm our finding that the DNA replication defect at interspersed repeat sequences, like LTR, coupled with error prone repair is the source of GIN caused by *mcm4*$^{Chaos3}$. Here I provide evidence that ploidy regulates the fragility of the interspersed repeat regions, which may explain the cell type specificity of GIN induced by replication stress. In metazoans, I envision that factors like the size of genome, the structure of chromatin, nuclear architecture, the amount of repetitive sequence, and the epigenetic state might determine the response to replication stress in different cell types.

In summary, this study extends beyond the question of whether replication stress causes GIN. I show that GIN is a potential, but not obligatory, outcome of replication stress. The challenge for the future will be to define different paths to replication stress, and how different cell types and different genetic contexts intertwine to determine whether replication stress leads to GIN.

**The LTR regions serve as fragile sites under ploidy regulation**

Ty elements are LTR-retrotransposons that replicate through an RNA intermediate and are representative of a class of mobile genetic elements existing in all eukaryotes. In *S. cerevisiae*, there are about 300 Ty related elements each flanked by LTRs clustered in about 30 - 40 locations (Gabriel et al. 2006). In addition, there is an order of magnitude more solo LTR elements (Gabriel et al. 2006). Altogether, these repetitive sequences represent about 3% of the genome. Ty elements encode about 10% of the total mRNA in haploid *Saccharomyces cerevisiae*, and alter the expression of the adjacent genes (Servant et al. 2008). It is known that most Ty elements are cell-type regulated, and their transcription is repressed in diploid cells (Company and Errede 1988; Fulton et al. 1988; Bilanchone et al. 1993; Morillon et al. 2000), which is partially if not totally regulated by *MAT* locus heterozygosity (Wilke et al. 1992).

A Ty element when recombines between LTRs delete the whole Ty element leaving a solo LTR as a "scar". The LTR regions contain a complex array of positively and negatively acting sequences. The Ty1 and Ty2 LTRs contain an upstream activation site (UAS) and two TATA sites (Liao et al. 1987). In the Ty5 LTR, there are several pheromone responsive elements (PRE) that are responsible for the transcriptional repression of Ty5 (Ke et al. 1997). The Ty3 LTR contains a negative control region, PRE, and a1/α2 binding sites (Bilanchone et al. 1993). The function of LTR as replication barriers in diploid demonstrated in Chapter 2 probably depends on the interaction of the proteins bound at these sites, and hence the local chromatin structure. Previous studies of retrotransposons have focused on the entire elements; here I showed that solo LTRs not only provide regions of portable homology for recombination, but also play an important role on the cell type specific response to replication stress.

Recently, it was shown that the expression of Ty is under RNA-dependent control from solo LTR regions (Jiang 2002; Berretta et al. 2008). Cosuppression is the high copy number-triggered silencing of dispersed homologous genes (Jorgensen 1995), which may evolve in eukaryotes to control molecular parasites such as viruses and transposons (Wolffe and Matzke 1999). Cosuppression is a fairly common process mainly through posttranscriptional gene silencing (RNAi) and transcriptional gene silencing. Such cosuppression also controls Ty1 expression and the promoter of Ty1 is required for this negative feedback control (Jiang 2002). Recently, it was found that an anti-sense cryptic transcript encompassing the Ty1 LTR mediates the silencing of Ty1, through a mechanism similar to heterochromatic gene silencing (Berretta et al. 2008). MCM genes seem to also involve in this cosuppression directly. It has been shown that *mcm5* mutants have upregulated expression of Ty proximal genes and defects in the establishment of compact chromatin domains near Ty elements (Dziak et al. 2003).

This raises the interesting hypothesis that the Ty regions may not be silenced in *mcm4*$^{Chaos3}$ diploid mutants and the change in chromatin structure might be a potential mechanism for LTR as fragile sites in diploid mutant.

**The tissue specificity of *MCM4*$^{Chaos3}$ induced cancer may be due to cell-type specific replication stress and repair pathways**

The striking tissue specificity of *MCM4*$^{Chaos3}$ mouse is similar to the previously reported *Brca1* and *Brca2* mutant. BRCA1 and BRCA2 mutations in humans confer a very high incidence of breast and ovarian cancer (Walsh and King 2007). A closer look at the functions of *Brca1, Brca2* and *Mcm4* indicates that these three genes all have functions converging on the fork. BRCA2 is a critical mediator of RAD51 (Walsh and King 2007) in stabilizing the stalled fork (Lomonosov et al. 2003) and in repairing of replication-mediated double-strand breaks generated when replication forks encounter interstrand cross-links (ICL) (Cipak et al. 2006). ICL is a unique class of DNA damage in which the two complementary strands of duplex DNA are covalently linked. This type of damage is intrinsically similar to a DNA replication helicase defect in which the two strands of DNA cannot be separated. Brca1 has a broader DNA repair function and interacts with Brca2 (Gudmundsdottir and Ashworth 2006). Brca1 is involved in both HR and NHEJ (Durant and Nickoloff 2005; Zhuang et al. 2006), and may regulate the repair pathway choice of DSBs degenerated from collapsed forks (Shrivastav et al. 2008b). Probably in the *Mcm4*$^{Chaos3}$ mammary gland tissue, the fork defect causes a higher demand for BRCA1/2 proteins for HR in restarting stalled or collapsed replication forks, which may explain the similar etiologies of tumors in these three mutants.

Despite intensive studies on BRCA1 and BRCA2, it is still unclear what is the mechanism for this tissue specificity (Monteiro 2003). The breast epithelium

proliferates rapidly during puberty and under the influence of estrogenic hormones. Similar to Ty element in yeast, Alu repeats, short interspersed elements, are the most abundant family of retroposons in human, a subset of which are still mobile and could cause genetic variability and heritable disorders (Schmid and Maraia 1992). A genome wide mapping of common fragile sites (CFSs) caused by oncogene-induced replication stress found CFS and regions with LOH enriched with Alu repeats (Tsantoulis et al. 2008), suggesting Alu elements may behave as fragile sites as LTR regions do. Interestingly, many Alu repeats contain a novel class of estrogen receptors (ERs) binding element with a high affinity for ERs that works as ER-dependent enhancers (Norris et al. 1995). The upregulation of the BRCA1 mRNA in human breast cells is mediated by this Alu-associated estrogen response element (ERE) in BRCA1 gene promoter (Tomilin 1999). These results, together with our finding on the cell type regulation of fragile sites in yeast, raise the interesting possibility that the binding of the estrogen receptor to the Alu sequence in mammary gland may cause stronger fragile sites than other tissues, making the mammary gland more vulnerable to mutant disrupting fork integrity.

**Haploid and Diploid yeast have fundamental differences**

The evolution of the sexual cycle that introduces a duplicate set of the genome, is believed to be driven by the need for greater protection from radiation (Cavalier-Smith 2002). Diploid cells are able to conduct HR regardless of cell cycle stages because of the presence of a homolog as template. In organisms with only one copy of the genome, such as *E.coli*, HR is a highly mutagenic process that is activated only under adverse conditions (Pennington and Rosenberg 2007). Many protozoa, algae, fungi, mosses, and ferns still maintain an alternation of generation with substantial development in haploid stage (Mable and Otto 1998). In haplophase of these species,

it is advantageous to have a mechanism to prevent inappropriate recombination when the homologous template is not available. However, little is known about how cells sense the existence of homologous copy and how HR is regulated during the ploidy change associated with sexual cycles.

Although diplophase is the dominant state in "higher" plants and animals (Valero et al. 1992), they still retain the mechanisms to prevent homologous recombination at certain stages. *Saccharomyces cerevisiae* is normally diploid in the wild. However, homolog search and strand invasion in the G1 phase is blocked by preventing the loading of RPA and Rad51 and by activating Mec1 (Ira et al. 2004). This suppression of HR depends on CDK1 activity (Ira et al. 2004), and similar regulation also occurs in mammals. The interaction of BRCA2 and RAD51 is blocked by CDK through phosphorylation of BRCA2 (Esashi et al. 2005) as one way to downregulate HR in M phase and G1 phase. However, such cell cycle regulation is overcome when cells were irradiated (Esashi et al. 2005), indicating multiple levels of regulation on appropriate HR.

The ploidy change also allows a more complex regulation of gene expression. Some species evolve a heteromorphic life cycle with the haploid and diploid phase targeting different ecological niches (Hannach and Santelices 1985). In yeast, diploid is specialized for meiosis and sporulation. Regardless of mating types, yeast has a ploidy dependent regulation of G1 cyclins and *FLO11* genes (Galitski et al. 1999b). It is reasonable to believe that higher eukaryotic cells still keep some of the mechanism of gene expression regulation by ploidy. The phenomena such as X-inactivation and genomic imprinting in diploid organisms, although its evolution significance is still unclear, could reflect the necessity for maintaining haploid levels of expression at key developmental stages (Mable and Otto 1998). Therefore, our study on the ploidy effect

probably eventually will trace back to gene expression regulation. Such expression regulation mechanism, and even the gene targets might be conserved.

As shown in our study, the dichotomous response to replication stress indicates a fundamental difference between haploid and diploid yeast. This will add another layer to the regulation evolved as a result of ploidy changes associated with sexual cycles.

**Materials and Methods**

*Yeast strains and plasmid*

Isogenic haploid W303 yeast strains *mcm4*$^{Chaos3}$ were constructed as described (Shima et al. 2007a). Strains used in this study are listed in Supplemental Table1. The *MAT*a/*Δ* and *MATΔ*/α diploid were constructed by disruptions of the *MAT* locus using pFP19 plasmid, a gift from Hannah Klein. Ectopic expression of NEJ1 was performed using PMV01 plasmid with empty vector PMV04 as control; both plasmids were requested from James Haber Lab.

*Flow cytometric analysis*

Approximately 1 x $10^7$ cells were collected from log-phase cultures and processed as described (Clarke et al. 2001). DNA was stained with Sytox Green (Molecular Probes, Eugene, OR) and profiles were analyzed using a Becton Dickinson (San Jose, CA) LSR II with a 530/30BP channel filter and BDFACSDiVa software Becton Dickinson (San Jose, CA).

*Growth curve and doubling time*

Saturated cell cultures were diluted 25 X in complete medium and then grown at 30°C for 4 hours to mid-log phase. The absorbance at 600 nm was measured every 30 minutes for 5 hours. The growth rates and doubling times were calculated during

exponential growth. For each experiment where doubling times of different strains are compared, all strains were processed simultaneously in at least two independent trials to yield variations in doubling times of less than 0.1 hr.

*Intrachromasomal recombination assay*

Each strain carried the recombination reporter *leu2-ri::URA3::leu2-bsteii*, which has a heteroallelic duplication of *LEU2*, with *URA3* between the *LEU2* genes. Gene conversion was determined by fluctuation tests, measuring Leu+ Ura+ rates. The deletion rate was determined by fluctuation tests, measuring fluoroorotic acid resistance rates. Each test was performed with ten colonies and done two times for each genotype (Xu et al. 2004).

*Determination of spontaneous mutation rates*

The forward mutation rate at the *CAN1* 1ocus was determined by standard methods (Sia et al. 1997), using at least 12 independent cultures for each rate estimate. Rates were calculated from the frequencies of canavanine-resistant mutants by using the method of the median (LEA and Coulson 1949).

*Measurement of GCRs*

The strains were conducted with marker on ~10 kb from the telomere of ChrXV-L to select for GCR events, and this GCR is probably due to the higher efficiency of BIR over de novo telomere addition in repairing DSBs. The GCR rate was measured based on the previous reported protocol (Kanellis et al. 2007). 10 colonies from each strain were tested, and two rounds of independent experiments were conducted.

*Comparative genomic hybridization (CGH) microarray*

Genomic DNA was prepared, sonicated and labeled based on the protocol from

the Dunham lab (Torres et al. 2007). DNA from the experimental strain was labeled with Cy3 nucleotide, and DNA from wild-type strain was labeled with a Cy5 nucleotide. In Figure 5, and 6, the wild-type haploid and widltype diploid are used as control respectively. The two DNA samples were mixed and hybridized to Yeast Whole Genome ChIP-on-chip Microarray from Agilent (290 nt resolution, 4 x 44K slide format, contains ~85% of the non-repetitive portion of the yeast genome, catalog #G4493A). Arrays were then washed according to the Agilent SSPE wash protocol, and scanned on an Agilent scanner. The image was processed using the default settings with Agilent Feature Extraction software. All data analysis was performed using the resulting log2 ratio data, and filtered for signals that are 2.5-fold above background in at least one channel.

## APPENDIX

### Supplemental Table 1. Strain list

| Strain | Genotype | Source |
|---|---|---|
| W303 | MATa *ade2-1 can1-100 his 3-11,15 leu2-3, 112 trp1-1 ura 3-1* | |
| XL47 | MATa *mcm4$^{Chaos3}$* | This lab |
| XL11 | MATa *leu2RI::URA3::leu2BstEII* | Derived from strain 1538- |
| XL12 | MATa *leu2RI::URA3::leu2BstEII mcm4$^{Chaos3}$* | 2C from Hannah Klein Lab |
| XL142 | MATa *CAN1* | Derived from MC42-2d |
| XL141 | MATa *CAN1 mcm4$^{Chaos3}$* | from Tom Pete lab |
| XL619 | MATα *ChrXV 10KB::CAN1-URA3* | Derived from strain |
| XL620, XL621 | MATα *ChrXV 10KB::CAN1-URA3 mcm4$^{Chaos3}$* | yDD1775 from Daniel Durocher Lab |
| XL10 | MATa *sml1::URA3 mrc1::HIS3* | Derived from strain |
| XL37 | MATa *sml1::URA3 mrc1::HIS3 mcm4$^{Chaos3}$* | YJT134 from John Diffley Lab |
| XL38 | MATα *sml1::URA3 mrc1::HIS3 mcm4$^{Chaos3}$* | |
| XL324 | MATa *mrc1-AQ::HIS3* | Derived from strain Y2298 |
| XL326 | MATa *mrc1-AQ::HIS3 mcm4$^{Chaos3}$* | from Hannah Klein Lab |
| XL327 | MATα *mrc1-AQ::HIS3 mcm4$^{Chaos3}$* | |
| XL178 | MATa *top3::LEU2* | Derived from strain |
| XL179 | MATa *top3::LEU2 mcm4$^{Chaos3}$* | KKY606-1A from Hannah Klein Lab |

**Supplemental Table 1 (Continued)**

| | | |
|---|---|---|
| XL54 | MATa *rad50::hisG-URA3-hisG* | Derived from strain |
| XL192 | MATα *rad50::hisG-URA3-hisG* | KKY604-17C from |
| XL193 | MATa *rad50::hisG-URA3-hisG* | Hannah Klein Lab |
| | *mcm4$^{Chaos3}$* | |
| XL194 | MATα *rad50::hisG-URA3-hisG* | |
| | *mcm4$^{Chaos3}$* | |
| XL94, | *rad50::null/rad50::null* | |
| XL95 | | |
| XL96, | *rad50::null/rad50::null* | |
| XL97 | *mcm4$^{Chaos3/Chaos3}$* | |
| XL236 | *rad52::TRP1/rad52::TRP1* | Derived from strain |
| XL237 | *rad52::TRP1/rad52::TRP1* | KKY614-10B from |
| | *mcm4$^{Chaos3/Chaos3}$* | Hannah Klein Lab |
| XL232 | MATa *sgs1::URA3* | Derived from strain |
| XL233 | MATa *sgs1::URA3 mcm4$^{Chaos3}$* | KKY1958-10A from |
| | | Hannah Klein Lab |
| XL336 | MATa *tof1::URA3* | Derived from strain |
| XL337 | MATα *tof1::URA3* | YHG307 from Rolf |
| XL338 | MATa *tof1::URA3 mcm4$^{Chaos3}$* | Sternglanz Lab |
| XL339 | MATα *tof1::URA3 mcm4$^{Chaos3}$* | |
| XL168 | MATα *rad6::LEU2* | Derived from strain M31 |
| XL170 | MATa *rad6::LEU2 mcm4$^{Chaos3}$* | from Takashi Hishida Lab |
| XL171 | MATa *rad6::LEU2 rad5* | |
| XL172 | MATa *rad6::LEU2 rad5 mcm4$^{Chaos3}$* | |

**Supplemental Table 1 (Continued)**

| | | |
|---|---|---|
| XL49 | MATα *rad51::HIS3* | Derived from strain |
| XL113 | MATα *rad51::HIS3 mcm4$^{Chaos3}$* | KHKY1039-4D from Hannah Klein Lab |
| XL126 | MATa *mgs1::LEU2 rad51::HIS3* | Derived from strain |
| XL137 | MATa *mgs1::LEU2 rad51::HIS3 mcm4$^{Chaos3}$* | TH201 from Takashi Hishida Lab |
| XL136 | MATα *mgs1::LEU2 rad51::HIS3 mcm4$^{Chaos3}$* | |
| XL134, XL135 | MATα *mgs1::LEU2 rad51::HIS3 rad5* | |
| XL129 | MATa *mgs1::LEU2 rad51::HIS3 rad5 mcm4$^{Chaos3}$* | |
| XL128 | MATα *mgs1::LEU2 rad51::HIS3 rad5 mcm4$^{Chaos3}$* | |
| XL157 | MATa *srs2::HIS3* | Derived from strain |
| XL158 | MATα *srs2::HIS3* | KKY590-1D from Hannah |
| XL159 | MATa *srs2::HIS3 mcm4$^{Chaos3}$* | Klein Lab |
| XL160 | MATα *srs2::HIS3 mcm4$^{Chaos3}$* | |
| XL299 | MATα *dnl4::URA3* | Derived from strain 1186- |
| XL300 | MATa *dnl4::URA3* | 5C from Hannah Klein |
| XL301 | MATa *dnl4::URA3 mcm4$^{Chaos3}$* | Lab |
| XL302 | MATα *dnl4::URA3 mcm4$^{Chaos3}$* | |
| XL303 | MATa *dnl4::URA3 rad52::TRP1* | |
| XL304 | MATa *dnl4::URA3 rad52::TRP1 mcm4$^{Chaos3}$* | |

**Supplemental Table 1 (Continued)**

| | | |
|---|---|---|
| XL305 | MATα *dnl4::URA3 rad52::TRP1 mcm4$^{Chaos3}$* | |
| XL16 | MATa *sml1::URA3 rad53::LEU2* | Derived from strain YJT75 |
| XL17 | MATa *sml1::URA3 rad53::LEU2 mcm4$^{Chaos3}$* | from John Diffley Lab |
| XL18 | MATa *sml1::URA3 mec1::LEU2* | Derived from strain YJT74 |
| XL19 | MATa *sml1::URA3 mec1::LEU2 mcm4$^{Chaos3}$* | from John Diffley Lab |
| XL20 | MATa *chk1::URA3* | Derived from strain |
| XL21 | MATa *chk1::URA3 mcm4$^{Chaos3}$* | DES220 from Steven Elledge Lab |
| XL161 | MATα *rad9::URA3* | Derived from strain 3834 |
| XL163 | MATα *rad9::URA3 mcm4$^{Chaos3}$* | from Judith Berman's lab |
| XL274 | MATα/Δ diploid | |
| XL276 | MATα/Δ diploid *mcm4$^{Chaos3/Chaos3}$* | |
| XL277 | MATa/Δ diploid | |
| XL280 | MATa/Δ diploid *mcm4$^{Chaos3/Chaos3}$* | |

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# CHAPTER 4

## The Pachytene Checkpoint and its Relationship to Evolutionary Patterns of Polyploidization and Hybrid Sterility

**Abstract**

Sterility is a commonly observed phenotype in interspecific hybrids. Sterility may result from chromosomal or genic incompatibilities, and much progress has been made toward understanding the genetic basis of hybrid sterility in various taxa. The underlying mechanisms causing hybrid sterility, however, are less well known. The pachytene checkpoint is a meiotic surveillance system that many organisms use to detect aberrant meiotic products, in order to prevent the production of defective gametes. I suggest that activation of the pachytene checkpoint may be an important mechanism contributing to two types of hybrid sterility. First, the pachytene checkpoint may form the mechanistic basis of some gene-based hybrid sterility phenotypes. Second, the pachytene checkpoint may be an important mechanism that mediates chromosomal-based hybrid sterility phenotypes involving gametes with non-haploid (either non-reduced or aneuploid) chromosome sets. Studies in several species suggest that the strength of the pachytene checkpoint is sexually dimorphic, observations that warrant future investigation into whether such variation may contribute to differences in patterns of sterility between male and female interspecific hybrids. In addition, plants appear to lack the pachytene checkpoint, which correlates with increased production of unreduced gametes and a higher incidence of polyploid species in plants versus animals. While the pachytene checkpoint occurs in many animals and in fungi, at least some of the genes that execute the pachytene checkpoint are different among organisms. This finding suggests that the penetrance of the

pachytene checkpoint, and even its presence or absence, can evolve rapidly. The surprising degree of evolutionary flexibility in this meiotic surveillance system may contribute to observed variation in patterns of hybrid sterility and in rates of polyploidization.

## Introduction

The genetic basis of postzygotic reproductive isolation has become clearer over the last decade. Depending on the specific mechanism involved, postzygotic isolation can be due to either genic or chromosomal incompatibility. In most animals, incompatibilities mediated by deleterious interactions among genes are thought to be the primary cause of hybrid inviability and sterility, as described by Dobzhansky (1936; 1937) and Muller (1940; 1942); see also (Orr and Turelli, 2001). In contrast, postzygotic isolation in plants often involves karyotypic changes caused by chromosomal rearrangement or genome duplication, i.e., polyploidy (Werth and Windham, 1991; Rieseberg *et al.*, 1995; Ramsey and Schemske, 1998; Comai, 2000; Lynch and Conery, 2000; Lynch and Force, 2000). However, little is known about the mechanistic basis for these incompatibilities. Here I discuss how a recent discovery from studies of meiotic regulation, the pachytene checkpoint, may contribute to our understanding of both chromosomal and gene-based hybrid sterility, and how the presence or absence of the pachytene checkpoint may be a critical factor in determining levels of polyploidization, a common route to speciation in plants. I also consider how sex-specific variation in the pachytene checkpoint may contribute to sex-specific patterns of variation in the penetrance of gene-based hybrid sterility.

## The pachytene checkpoint

It is critical that the chromosomes carried by sperm, eggs and spores contain accurate representations of the parental genome. During meiosis, the maternal and

paternal copies of each chromosome align with each other and form a synaptonemal complex, exchange genetic material via the process of recombination, and separate to daughter cells in the first of two meiotic cell divisions. These intricate chromosomal events are subject to errors, so most organisms have evolved meiotic "checkpoints" that monitor the fidelity of chromosome synapsis and the repair of DNA damage. These checkpoints cause defective cells to self-destruct, thus preventing the generation of defective gametes.

Pachytene is the stage of meiotic prophase I where chromosomes are completely aligned with their homologous partner, a process known as synapsis. In many species, including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans* and mice, defects in recombination and/or synapsis lead to arrest during the pachytene stage, and arrested cells will eventually be eliminated (Bishop *et al.*, 1992; Sym *et al.*, 1993; Edelmann *et al.*, 1996; McKee and Kleckner, 1997; Chua and Roeder, 1998; Pittman *et al.*, 1998; Ghabrial and Schupbach, 1999; Gartner *et al.*, 2000). This surveillance process is referred to as the pachytene checkpoint. For organisms with a strict pachytene checkpoint, triploid or trisomic individuals cannot generate gametes, and meiotic cells with pairing or recombination defects are aborted.

**Evidence for different mechanisms of the pachytene checkpoint in different species**

While the pachytene checkpoint is observed in many organisms ranging from yeast to mammals, it appears that different mechanisms may be involved in different species. It has been suggested that there are at least two surveillance mechanisms that monitor meiotic chromosome metabolism in budding yeast, worms and mice. One pathway monitors recombination intermediates, and the other monitors incomplete

synapsis. Pachytene checkpoint genes have been most intensively studied in the budding yeast *S. cerevisiae* (reviewed in Lee and Amon, 2001). Numerous mutations have been identified that block chromosome synapsis and/or recombination and induce pachytene arrest (Roeder, 1997; Roeder and Bailis, 2000). The characterization of secondary mutations that bypass pachytene arrest in the presence of defects in recombination and synapsis has led to the identification of pachytene checkpoint genes (reviewed in Roeder and Bailis, 2000). For example, in *S. cerevisiae*, *PCH2* was identified in a genetic screen for mutations that bypass the pachytene arrest triggered by deletions of genes such as *ZIP1* (which encodes a synaptonemal complex central element component) (San-Segundo and Roeder, 1999). In this species, several genes involved in the mitotic DNA damage checkpoint also participate in the pachytene checkpoint to monitor the presence of recombination intermediates (Lydall *et al.*, 1996; Leu and Roeder, 1999; San-Segundo and Roeder, 1999; Hochwagen *et al.*, 2005; Hochwagen and Amon, 2006). However, in mammals, the somatic checkpoint and pachytene checkpoint might use different genes. For example, *Atm*-deficient somatic cells are highly resistant to radiation-induced apoptosis (Xu and Baltimore, 1996), while meiocytes in *Atm*-null mice can still trigger the pachytene checkpoint and undergo apoptosis (Barlow *et al.*, 1998).

Genetic studies of *S. cerevisiae* and the nematode *C. elegans* have indicated that *PCH2* is required for a checkpoint that monitors defects in synapsis, but not DNA damage (Bhalla and Dernburg, 2005; Wu and Burgess, 2006). *PCH2* orthologs have been found in many organisms (Wu and Burgess, 2006; Li and Schimenti, 2007), suggesting that synaptic checkpoints might be conserved across taxa. However, recent work indicates that the *PCH2* ortholog (called *Trip13*) in mice seems to not play a role in pachytene checkpoint control (Li and Schimenti, 2007). Furthermore, most of the other synapsis checkpoint genes identified in yeast do not have orthologs in mammals.

These findings suggest that the "pachytene checkpoint" has evolved independently in different organisms.

**Evidence for differences in the strictness of the pachytene checkpoint in different sexes**

Mouse meiotic mutants exhibit significant sexual dimorphism attributable to differential sensitivity of meiotic checkpoints (Hunt and Hassold, 2002). Spermatocytes are sensitive to mutations that disrupt either recombinational double strand break (DSB) repair or synapsis. In contrast, while oocytes are efficiently eliminated in response to unrepaired DNA damage, they are less sensitive to incomplete synapsis (Yuan *et al.*, 2000; Hunt and Hassold, 2002; Barchi *et al.*, 2005; Di Giacomo *et al.*, 2005).

Sexual dimorphism has also been observed in triploids. Interestingly, triploid chickens (which have ZW chromosomes) have reversed sexual dimorphism compared to mice (which have XY chromosomes). In triploid chickens, most oocyte loss occurs in young females a few weeks after hatching, probably as a consequence of the pachytene checkpoint. However, spermatocytes from male triploids can complete meiosis without a delay. While these spermatocytes do not form fully functional sperm they do reach the advanced stage of round spermatids (Lin *et al.*, 1986).

The frog, *Buergeria buergeri* (Anura, Rhacophoridae), also has a ZW sex chromosome system and appears to have a less strict meiotic checkpoint in males. Ohta *et al*. (1999) found that all ZWW-type triploid frogs were females having small ovaries, and oocytes were scarce and small. However, there was little difference in the number of germ cells compared to normal diploid females, suggesting that a checkpoint is blocking meiosis. In contrast, all ZZZ-type triploid frogs were males, and their testes produced spermatozoa. These spermatozoa did show some minor

defects, being somewhat long and thick when compared to spermatozoa produced by diploid males (Ohta *et al.*, 1999). Nevertheless, the presence of spermatozoa indicates that meiosis was not aborted in these triploid males.

Since asynapsis normally triggers the pachytene checkpoint, the heterogametic sex with distinctive heteromorphic sex chromosomes must evolve a mechanism to suppress detection of asynapsis. The failure of such a mechanism would trigger the checkpoint only in the heterogametic sex, which may underlie the examples of sexual dimorphism described above. During meiotic prophase in male mammals, meiotic sex chromosome inactivation (MSCI) causes the X and Y chromosomes to be incorporated into the heterochromatinized XY body (also called the sex body) (Handel, 2004; Turner *et al.*, 2006). It has been suggested that the failure of MSCI may trigger cell death in defective spermatocytes (Turner *et al.*, 2005), since in most meiotic mutants and sterile hybrid mice, the formation of the XY body is disrupted (Fernandez-Capetillo *et al.*, 2003; Turner *et al.*, 2005; Homolka *et al.*, 2007). In ZW female birds and snakes, although the W chromosome is not silenced, it has been shown that the unpaired regions are thickened and become partially heterochromatic (Forejt and Gregorova, 1977; Becak and Becak, 1981). Both the MSCI of the XY chromosomes and heterochromatinization of the remaining unpaired ZW regions may provide the mechanisms to suppress their intrinsic asynapsis (Jablonka and Lamb, 1988). Organisms with homomorphic sex chromosome do not face an intrinsic asynapsis problem, so they may not have a stricter pachytene checkpoint in the heterogametic sex. If so then I would predict that sexual dimorphism in the degree of sterility in triploids will be more pronounced in species which have older, more differentiated sex chromosomes.

These examples bear similarity to Haldane's Rule, which describes the observation that the heterogametic sex (XY or ZW) is more likely to show hybrid lethality or sterility than the homogametic sex (XX or ZZ) (Haldane, 1922). Haldane's Rule is likely to reflect several underlying causes, including the recessivity of hybrid incompatibility genes, the higher accumulation of such genes on the X chromosome, and the higher rate of evolution of male versus female hybrid sterility genes (Wu and Davis, 1993; Masly and Presgraves, 2007). Because the last explanation would counteract Haldane's Rule in ZW species where females are preferentially sterile or lethal, more research is needed to understand the mechanistic basis of sterility in ZW females of species such as birds (Price and Bouvier, 2002) and Lepidoptera (Presgraves, 2002). To this end, I suggest that sexual dimorphism in the pachytene checkpoint is one promising direction for future research.

However, I note that even if sexual dimorphism is found in additional ZW species it cannot be the sole determinant of Haldane's Rule. Haldane's Rule does not hold in diploid *Xenopus*, with all described interspecific crosses producing sterile hybrid males and fertile hybrid females (Kobel, 1996). Therefore, if male *Xenopus* do have a weaker pachytene checkpoint, as the data from triploid taxa suggest, then this difference is clearly not causing the sex-specific differences in hybrid sterility. The violation of Haldane's Rule may reflect the fact that most amphibian species do not have morphologically distinct sex chromosomes (Eggert, 2004). A recent study suggests that this preferential male sterility is caused by spermatogenesis being more sensitive to disruption in hybrids compared to oogenesis (Malone and Michalak, 2008). This finding is consistent with an earlier hypothesis for why males evolve hybrid sterility faster than females (Wu and Davis, 1993). It will be interesting to evaluate at which stage spermatogenesis is blocked in the *Xenopus* hybrid males and to determine whether they have a meiotic or post-meiotic defect.

**The pachytene checkpoint contributes to the speciation process by preventing hybrids from reproducing**

While many cases of sterile hybrids have been described, the underlying causes of sterility have typically been investigated using classical genetic or cytological methods only, or remain entirely unknown. Indeed, the pachytene checkpoint has only recently been described and investigated on a molecular level. Here, I describe a few examples of hybrid sterility that may reflect the activity of the pachytene checkpoint. When doing so, it is important to distinguish between the terms "sterile" and "infertile." Sterile refers only to situations in which no gametes are produced, while infertile refers to situations in which gametes are produced, but they are non-functional (or sub-functional). These phenotypes are likely triggered by different mechanisms. Sterility of hybrids may potentially result from activation of the pachytene checkpoint, while infertility of hybrids is more likely due to other types of surveillance mechanisms, such as those that sense dosage effects due to aneuploidy or structural defects in gametes. I suggest two ways in which consideration of the pachytene checkpoint may be helpful in understanding hybrid sterility: The first is as a mechanism that may be triggered by the action of hybrid incompatibility genes. I emphasize that there is unlikely to be a single mechanistic explanation for genic HI phenotypes. The second may reflect a more central role of the pachytene checkpoint in sensing chromosomal incompatibilities.

**The pachytene checkpoint and genic incompatibilities**

Hybrid sterility in mammals has been well-studied in rodents (Forejt, 1996; Borodin *et al.*, 1998; Borodin *et al.*, 2006), where it shows a clear pattern of pachytene checkpoint effects. Female hybrids are mostly fertile, while male hybrids are sterile with meiotic arrest around pachytene (Forejt and Ivanyi, 1974; Yoshiki *et al.*, 1993).

Aberration of chromosome pairing has been detected at the pachytene stages of hybrid male mice (Chandley, 1988). Apparently, asynapsis in the hybrids triggers the checkpoint effects.

Nevertheless, detailed studies indicate that genic incompatibility also contributes to hybrid sterility. The ability of certain chromosome rearrangements to pass through meiosis depends on the genetic background (de Boer and de Jong, 1989; Speed, 1989). In hybrids between two races of the house shrew *Suncus murinus* that differ by five Robertsonian translocations, heterozygotes for each of the translocation chromosomes that distinguish the two parental races were found in both sterile and fertile males (Borodin *et al.*, 1998), suggesting that chromosomal differences cannot be the sole cause of sterility. In another study, there was no apparent pairing defect in the male hybrids between *Thrichomys apereoides subsp. apereoides* and *T. apereoides subsp. laurentius*, but no spermatocytes beyond diplotene were detected, suggesting that the pachytene checkpoint is activated. This meiotic disruption phenotype segregates during backcrossing in males with homomorphic karyotypes, again suggesting a genic rather than chromosomal basis for sterility (Borodin *et al.*, 2006). So far, five hybrid sterility (*Hst*) loci have been identified in the mouse genome (Forejt, 1996), but the genes have not been identified. *Hst1*-dependent sterility causes arrest at the pachytene stage (Forejt, 1996), which suggests pachytene checkpoint pathways may be involved. It also might be the case that only certain regions in the genome, possibly the *Hst* loci, work as sensors to monitor the pairing, and are able to trigger the pachytene checkpoint. Other hybrid male sterility phenotypes appear to manifest post-meiotically (Good *et al.*, 2008), so pachytene arrest is clearly not the sole cause of mouse hybrid sterility.

**The pachytene checkpoint and chromosomal incompatibilities**.

Chicken-pheasant hybrids are viable but sterile with a meiotic disruption. Stages beyond primary spermatocytes are not present, indicating the likely role of the pachytene checkpoint in hybrid sterility (Purohit and Basrur, 1977). Cytological studies indicate that the Z chromosomes of chickens and pheasants appear to be identical between the species, while the autosomal complements are radically different (Stenius *et al.*, 1963), suggesting that chromosomal incompatibilities are responsible for the hybrid sterility.

Two well-known examples of sterile hybrids are hinnies, from crossing male horses with female donkeys, and mules, from crossing female horses with male donkeys. Karyotypic dissimilarities exist between the horse (64 chromosomes), the donkey (62 chromosomes), and the ensuing hybrids (63 chromosomes), helping to explain why hybrids are typically sterile. The first mechanistic explanation of a male mule's sterility was provided by Wodsedalek (1916) who concluded that there was a block in meiosis. More recent cytological studies by electron microscopy of the mule testes have shown that spermatogenesis can only reach the pachytene stage, at which time degenerative changes occur (Hernández-Jáuregui and Márquez Monter, 1977). In female mules and hinnies, there is also a severe depletion of oocytes in the ovaries, with most oocytes being rapidly eliminated after birth (Taylor and Short, 1973), which is around the pachytene stage. These data strongly suggest that meiocytes in mules are eliminated by the mammalian pachytene checkpoint. Interestingly, there is also a block in spermatogenesis at the pachytene stage of meiosis in the testes of zebra-horse and zebra-donkey hybrids, where the karyotype differences between the parental species are even greater (King *et al.*, 1965).

In reptiles and fish, hybrid triploids are often formed and the triploid females

are sterile. For example, triploids induced in the hybrid sturgeon (*Huso huso* female crossed to *Acipenser ruthenus* male) are sterile because they are unable to complete meiosis (Omoto *et al.*, 2005). Another example is the diploid unisexual lizard *Aspidoscelis dixoni*. *A. dixoni* normally reproduces parthenogenetically but it can mate with males from the bisexual species *A. tigris punctilinealis*. The resulting triploid female hybrids are sterile (Cole *et al.*, 2007). Interestingly triploid parthenogenetic species have evolved multiple times within this genus (Reeder *et al.*, 2002). For example, in triploid *Cnemidophorus uniparens* females the pachytene checkpoint is overcome by premeiotic endoduplication (Cuellar, 1971). This override presumably occurs because after premeiotic endoduplication the 6N meiocytes are able to synapse better than 3N meiocytes.

**The absence of the pachytene checkpoint - a potential factor contributing to the high frequency of polyploidization in plants**

The pachytene checkpoint has not been found in plants. Most plant meiotic mutants complete meiosis and cytokinesis and produce abnormal microspores (Caryl *et al.*, 2003). It is uncertain whether the pachytene checkpoint was initially present and then lost during plant evolution, though it does not occur in at least one basal species, the green algae *Chlamydomonas reinhardtii.* Triploid *C. reinhardtii* zygotes formed from a haploid-diploid mating can produce viable spores (Dutcher, 1988).

A major consequence of the lack of a pachytene checkpoint in plants is that, in contrast to the frequent sterility of hybrid animals, hybrid plants are generally able to produce gametes. However, a relatively large proportion of gametes produced by hybrid plants are either unbalanced (leading to infertility) or unreduced (i.e., containing the somatic chromosome number). Indeed, the mean estimates of unreduced gamete production are as high as 27.52% in hybrid taxa compared with

0.56% in nonhybrids (Ramsey and Schemske 1998). As Levin (2002) points out, this represents a nearly 50-fold increase in the production of unreduced gametes among hybrid relative to nonhybrid taxa, and is likely due to a high incidence of meiotic irregularities in the former relative to the latter. The production of unreduced gametes can result in decreased fertility when most conspecifics are producing normal, reduced gametes. In this situation, the reduction in fertility occurs because the fusion of unreduced (e.g., 2n) and reduced (e.g., 1n) gametes produces zygotes with uneven chromosome numbers (e.g., 3n). However, if multiple individuals within a population are producing unreduced gametes, or if a single individual producing unreduced gametes reproduces via self-fertilization, viable zygotes containing twice the number of chromosomes as their parents (i.e., polyploids) can be formed.

Polyploidy can arise through the doubling of chromosomes in somatic cells or through the union of unreduced gametes (deWet 1980; Ramsey and Schemske 1998; Levin 2002). For many years it was believed that somatic chromosome doubling was the predominant mode of polyploid formation, because the production of unreduced gametes was thought to be relatively rare. However, I now know that the production of unreduced gametes is more common than previously believed, and that their union is a common route to the evolution of polyploid lineages (Harlan and deWet 1975; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). Unreduced gametes can arise due to errors at several points during meiosis, but the majority occur after pachytene. Therefore, in terms of its influence on the evolution of polyploidy in plants, the lack of the pachytene checkpoint is probably most important when unreduced gametes arise as a result of what Lelley et al. (1987) and Bretagnolle and Thompson (1995) call "premeiotic doubling". For this reason, the lack of the pachytene checkpoint might provide one of several distinct mechanistic explanations for the ability of plants to initially establish polyploidy at high frequency relative to

other taxonomic groups. In conclusion, the lack of a pachytene checkpoint can potentially contribute to polyploidy in two ways. First, it allows the survival of unreduced gametes from premeiotic doubling (or other events that produce unreduced gametes before or during pachytene). Second, it allows newly formed polyploids and hybrids to produce gametes, facilitating the maintenance and long-term persistence of polyploid populations.

The absence of a pachytene checkpoint may also promote intraspecific variation in plants compared to animals by increasing the tolerance for sequence divergence. In animals, the pachytene checkpoint effectively prevents interspecific gene flow. For plants, however, meiosis can proceed in hybrids even if relatively high sequence divergence causes asynapsis between chromosomes from the two parental strains or species. Thus the absence of a pachytene checkpoint may help to explain high levels of variation in some plants, for example, the finding that two lineages of maize are, on average, as genetically diverged as humans and chimpanzees (Buckler et al. 2006).

**Do animal polyploids lack the pachytene checkpoint?**

Polyploidy is relatively rare in most groups of animals. However, there is increasing evidence that in some groups of animals polyploidy is more frequent than previously believed (Bogart 1980; Lokki and Saura 1980; Schultz 1980; Otto and Whitton 2000; Gallardo et al. 2004; Le Comber and Smith 2004; Mable 2004). Because the pachytene checkpoint effectively eliminates the production of unreduced gametes in animals, I might expect that, relative to plants, a higher proportion of polyploid animals are formed via mitotic duplication (i.e., endoduplication). Interestingly, this prediction is consistent with the observation that the majority of polyploid animals reproduce parthenogenetically, and the evolution of parthenogenesis in animals typically predates polyploidy (Suomalainen et al. 1987; Otto and Whitton

2000). These findings raise two major questions. First, what accounts for the general difference in rates of polyploidization between plants and animals? Second, what accounts for the relatively high incidence of polyploidy in certain animal groups?

A number of explanations have been suggested to account for these patterns (Muller 1925; Orr 1990; Mable 2004). I suggest that the lack of a pachytene checkpoint in plants is an additional explanation for the first question of why polyploidy is generally more common among plants. How do I account for the presence of polyploid animals among some insects, reptiles, amphibians and fish? One possibility is that these polyploid animals have lost the pachytene checkpoint genes that are present in their non-polyploid sister taxa. This scenario would imply that polyploid animals would proceed through meiosis even when subject to various meiotic defects. Indeed, as I have noted, the pachytene checkpoint is sexually dimorphic in some species, which demonstrates that variability in the penetrance of the pachytene checkpoint can be tolerated within species.

Alternatively, these animal polyploids may have evolved mechanisms to override the ability of the pachytene checkpoint to detect or respond to the presence of non-haploid gametes, as in the case of premeiotic endoduplication. This scenario would also imply that polyploids retain the ability to arrest meiotic products that have received other types of DNA damage. Analysis of the pachytene checkpoint in the model organisms zebrafish (*Danio rerio*) and Xenopus frogs (*Xenopus laevis*) will provide useful data for launching comparative studies of polyploid fish and amphibians to investigate whether and how they have evolved the ability to produce non-haploid gametes.

In conclusion, the pachytene checkpoint is not only a surveillance system in meiosis. I suggest that the presence or absence of a pachytene checkpoint has a profound influence on the evolutionary strategies of different eukaryotic groups, and

of the different sexes. Based on evidence reviewed here I can make two conclusions. First, the pachytene checkpoint is the underlying mechanism for hybrid sterility in some cases, but more cytological evidence on additional hybrids is required to assess the generality of this finding. Second, the absence of a pachytene checkpoint in plants might influence the production and/or survival of unreduced gametes and allow newly formed polyploids and hybrids to produce viable gametes, facilitating the maintenance and long-term persistence of polyploid populations. Further research on polyploid animals is one direction that I suggest will help to test this hypothesis. The finding that different organisms appear to use different genes to execute the pachytene checkpoint underscores the evolutionary lability of this meiotic surveillance system, and calls for the further study of the molecular mechanisms controlling the pachytene checkpoint in different organisms.

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CHAPTER 5

# Mouse Pachytene Checkpoint 2 (Trip13) Is Required for Completing Meiotic Recombination but Not Synapsis


## Abstract

**Background:** In mammalian meiosis, homologous chromosome synapsis is coupled with recombination. As in most eukaryotes, mammals meiocytes have checkpoints that monitor the fidelity of these processes.

**Findings:** I report that the mouse ortholog (*Trip13*) of pachytene checkpoint 2 (*PCH2*), an essential component of the synapsis checkpoint in *S. cerevisiae* and *C. elegans*, is required for completion of meiosis in both sexes. TRIP13-deficient mice exhibit spermatocyte death in pachynema and loss of oocytes around birth. The chromosomes of mutant spermatocytes synapse fully, yet retain several markers of recombination intermediates, including RAD51, BLM, and RPA. These chromosomes also exhibited the chiasmata markers MLH1 and MLH3, and okadaic acid treatment of mutant spermatocytes caused progression to metaphase I with bivalent chromosomes. Double mutant analysis demonstrated that the recombination and synapsis genes *Spo11*, *Mei1*, *Rec8* and *Dmc1* are all epistatic to *Trip13*, suggesting that TRIP13 does not have meiotic checkpoint function in mice.

**Conclusion:** Our data indicate that TRIP13 is required after strand invasion for completing a subset of recombination events, but possibly not those destined as

crossovers. To our knowledge, this is the first model to separate recombination defects from asynapsis in mammalian meiosis, and provides the first evidence that unrepaired DNA damage alone can trigger the pachytene checkpoint response in mice.

## INTRODUCTION

The genesis of gametes containing an intact, haploid genome is critical for the prevention of birth defects, and is highly dependent upon the fidelity of chromosome dynamics before the first meiotic division. Homologous chromosomes must pair, synapse, undergo recombination, and segregate properly to opposite poles. Recombination, which repairs repair double strand breaks (DSBs) that are genetically-induced in leptonema, is coupled with synapsis in budding yeast and mammals. While our knowledge on the assembly and nature of recombination machinery is extensive, little is known about the disassembly of recombination intermediates, recruitment of DNA replication machinery during recombinational repair, and how the choice between different repair pathways is made.

Defects in recombination can preclude homologous chromosome pairing, leave unrepaired chromosome breaks, and cause aneuploidy by abrogating crossing over. To avoid such deleterious outcomes, surveillance systems ("checkpoints") exist to sense meiotic errors and eliminate cells containing unresolved defects. In many organisms, including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans* and mice [1-4], meiocytes with defects in recombination and/or chromosome synapsis trigger meiotic arrest in the pachytene stage of meiotic prophase I. This response to meiotic defects is referred to as the "pachytene checkpoint" (reviewed in [5]). Genetic experiments in *S. cerevisiae* have identified elements of the pachytene checkpoint machinery (reviewed in [5]). In addition to

meiosis-specific proteins, these include factors that play roles in DNA damage signaling in mitotic cells [6-10]. *Arabidopsis thaliana* does not appear to have a pachytene checkpoint akin to that in yeast [11], nor do male *Drosophila*.

The pachytene checkpoint is known to monitor two aspects of meiotic chromosome metabolism in *S. cerevisiae* and *C. elegans*: 1) DSB repair, and 2) chromosome synapsis [2,12]. In mice, both spermatocytes and oocytes harboring mutations that disrupt DSB repair (such as *Dmc1, Msh5,* and *Atm*) are efficiently eliminated in pachynema, but spermatocytes are much more sensitive to DSB repair-independent synapsis defects than oocytes [13-15]. However, because recombination is required for synapsis in mice (mutations in recombination genes such as *Dmc1* cause extensive asynapsis [16]), it has remained formally uncertain whether there is a distinct pachytene checkpoint that responds to defects in meiotic recombination, and if so, whether it would be identical to that used in somatic cells. The mechanisms of putative pachytene checkpoint control remain unknown in mammals, since no mutations have been identified that abolish it.

*PCH2*, encoding a nucleolar-localized AAA-ATPase that was originally identified in an *S. cerevisiae* genetic screen for mutants that relieve pachytene arrest of asynaptic *zip1* mutants [8], was recently determined to be an essential component of the pachytene synapsis (but not DSB repair) checkpoint in yeast and worms [2,12]. *PCH2* orthologs are present in organisms that undergo synaptic meiosis, but not asynaptic meiosis, prompting the suggestion that a Pch2-dependent checkpoint evolved to monitor synaptonemal complex (SC) defects from yeast to humans [12]. Here, we generated mice deficient for the *Trip13*, the ortholog of *PCH2*, and evaluated whether it also plays a role in the pachytene checkpoint. Surprisingly, while I found no evidence for checkpoint function, I did uncover a potential role for this protein in noncrossover (NCO) repair of meiotic DSBs.

## RESULTS

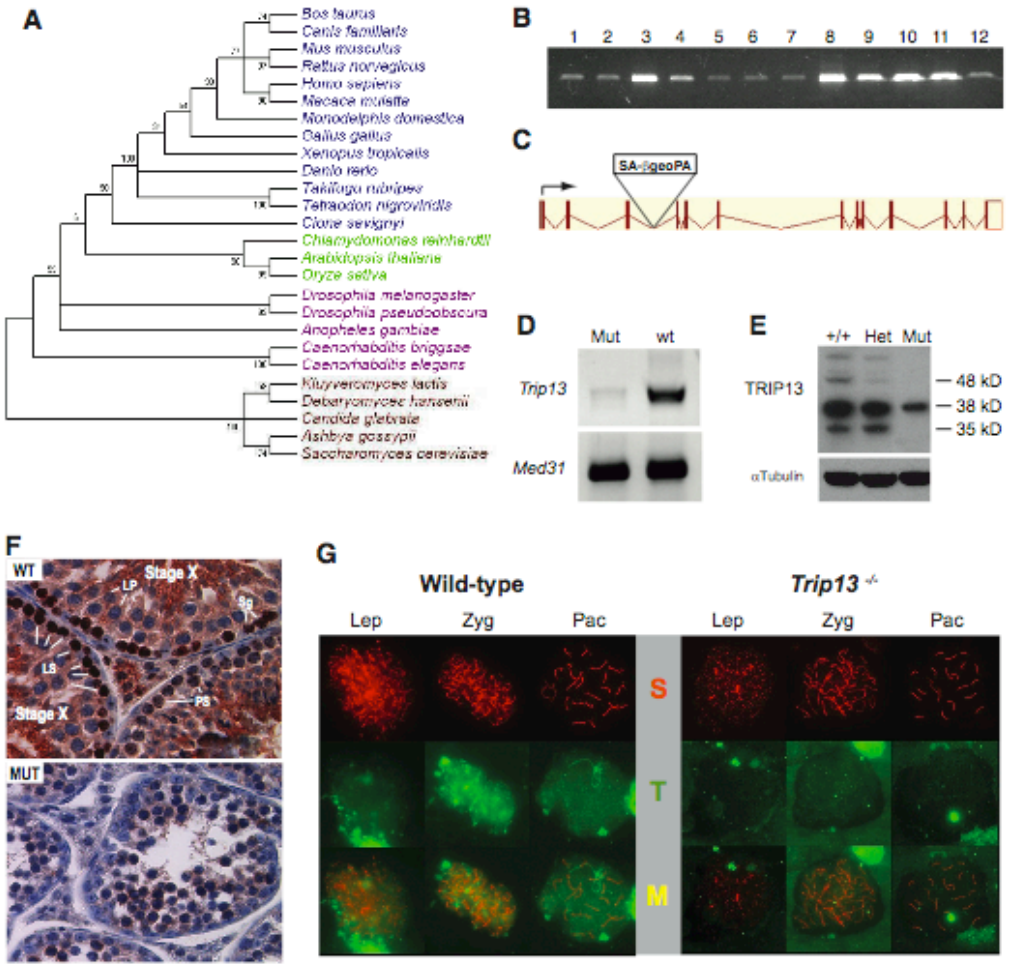
### *Trip13* is a widely-expressed mammalian ortholog of *PCH2* with unusual phylogenetic relationships

The mammalian ortholog of *PCH2*, *Trip13 (*Thyroid hormone receptor interacting protein 13), encodes a protein with extensive amino acid homology in regions alignable to the yeast and worm orthologs (Figure S1)[12]). Interestingly, phylogenetic analysis of TRIP13/Pch2p shows that the mammalian protein clusters more closely to plants than it does to the evolutionarily more closely related worms and flies (Figure 1A; see Discussion). Semi-quantitative RT-PCR analysis showed *Trip13* mRNA to be expressed in a variety of embryonic and adult tissues, including testis (Figure 1B), consistent with mouse and human EST data summarized in Unigene (www.ncbi.nlm.nih.gov/UniGene). It is also highly expressed in human and mouse oocytes [17].

### Generation of *Trip13* mutant mice

To explore the function of TRIP13 in mammals, we generated mice with a gene trap-disrupted allele, $Trip13^{RRB047}$ (Figure 1C; abbreviated as $Trip13^{Gt}$). Heterozygotes were normal in all respects, but homozygotes were present at ~2/3 the expected ratio from intercrosses between heterozygotes (91 $Trip13^{+/+}$ : 183 $Trip13^{Gt/+}$ : 61 $Trip13^{Gt/Gt}$). Since >90% of prewean mice that died were mutant homozygotes, this discrepancy is apparently due to a partially penetrant lethality. Most surviving $Trip13^{Gt/Gt}$ animals were grossly normal. However, homozygotes that were semi-congenic (N4) on the C57BL/6J strain were often markedly smaller and/or had kinked or shorter tails (Figure 2A,B).

**Figure 1.** The mouse PCH2 ortholog TRIP13 and expression in wild-type and mutant. **(A)** Phylogenetic tree of presumed PCH2/TRIP13 orthologs. The database sequence ID of each protein is presented in Table S1. Numbers shown are bootstrap values (see Methods). Major eukaryotic groups are indicated in color, with deuterostomia in blue, plants in green, protostomia in purple and fungi in maroon. **(B)** Amplification products of cDNA from the following tissues: 1=heart, 2=brain, 3=spleen, 4=lung, 5=liver, 6=skeletal muscle, 7=kidney, 8=testis, 9=E7 embryo, 10=E11 embryo, 11=E15 embryo, 12=E17 embryo. **(C)** Intron-exon structure of TRIP13 and insertion site of gene-trap vector. See Methods online for details on how the precise insertion site was identified. **(D)** RT-PCR of *Trip13* and a control gene Med31 from testis RNA. The *Trip13* primers are situated in the first and last exons (see Methods). **(E)** Western blot analysis of testis protein with anti-TRIP13 antibody. The blot was later probed with anti-alpha tubulin actin as a loading control. The expected TRIP13 protein is ~48KD. **(F)** Localization of TRIP13 in testes. Wild-type (top) and mutant (bottom) testis sections were probed with chicken anti-TRIP13, and detected with HRP-conjugated anti-chicken IgG (brown/red staining). Expression in WT was most prominent in the nuclei of Type B spermatogonia (Sg), leptotene spermatocytes (LS) and early pachytene spermatocytes (PS), but not late pachytene spermatocytes (LP). No nuclear staining was seen in mutant testis sections, although reddish cytoplasmic background is present. Identification of cell types was judged in part by estimating the epithelial stage of the tubules as described [67]. **(G)** TRIP13 localization in surface-spread spermatocytes. Preparations were immunolabelled with anti-SYCP3 (S) and TRIP13 (T). Both individual and merged images are shown for leptotene (Lep), zygotene (Zyg) and pachytene (Pac) spermatocytes. Nuclear staining was absent in the mutant.


A
Bos taurus
Canis familiaris
Mus musculus
Rattus norvegicus
Homo sapiens
Macaca mulatta
Monodelphis domestica
Gallus gallus
Xenopus tropicalis
Danio rerio
Takifugu rubripes
Tetraodon nigroviridis
Ciona savignyi
Chlamydomonas reinhardtii
Arabidopsis thaliana
Oryza sativa
Drosophila melanogaster
Drosophila pseudoobscura
Anopheles gambiae
Caenorhabditis briggsae
Caenorhabditis elegans
Kluyveromyces lactis
Debaryomyces hansenii
Candida glabrata
Ashbya gossypii
Saccharomyces cerevisiae
B
1 2 3 4 5 6 7 8 9 10 11 12
C
SA-βgeoPA
D
Mut wt
Trip13
Med31
E
+/+ Het Mut
TRIP13
48 kD
38 kD
35 kD
αTubulin
F
WT
Stage X
LP
Sg
LS
PS
Stage X
MUT
G
Wild-type
Trip13 -/-
Lep Zyg Pac
Lep Zyg Pac
S
T
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RT-PCR analysis of *Trip13*$^{Gt}$ expression (Figure 1D) revealed a low level of normally spliced transcripts in testes of homozygotes that is presumably a consequence of incomplete usage of the gene trap's splice acceptor. Western blot analysis, using a polyclonal antibody raised against a peptide encoded by exon 3, revealed multiple species in wild-type and heterozygous testes, one of which corresponds to the expected size of 48 kD (Figure 1E). This and 3 other species were undetectable in homozygous mutant testes, but a reduced amount of an intense ~38 kD smaller band was present. It is not clear if this corresponds to TRIP13. The greatly decreased *Trip13* mRNA and predicted correct-length protein in mutants indicate that the *Trip13*$^{RRB047}$ allele is severely hypomorphic.

To determine the germ cell types in which TRIP13 is expressed, and to assess possible expression in the mutant by means other than Western analysis, testis sections were immunolabelled for TRIP13 using a polyclonal chicken antipeptide antibody (see Methods). The most intensely labeled cells in control testes were Type B spermatogonia and leptotene spermatocytes (Figure 1F). Zygotene/pachytene spermatocytes stained less strongly, and there was no detectable staining in late pachytene spermatocytes. TRIP13 appeared to be nuclear localized. There was no such staining of nuclei in mutant seminiferous tubules (Figure 1F). To further assess the nuclear localization, TRIP13 was used to probe meiotic chromosomes prepared by surface spreading of spermatocyte nuclei. In wild-type, there was diffuse nuclear staining, and no evidence of concentration on synaptonemal complex cores (marked by the axial element protein SYCP3) at any meiotic substage (Figure 1G). TRIP13 signal was noticeably absent in mutant meiotic nuclei.

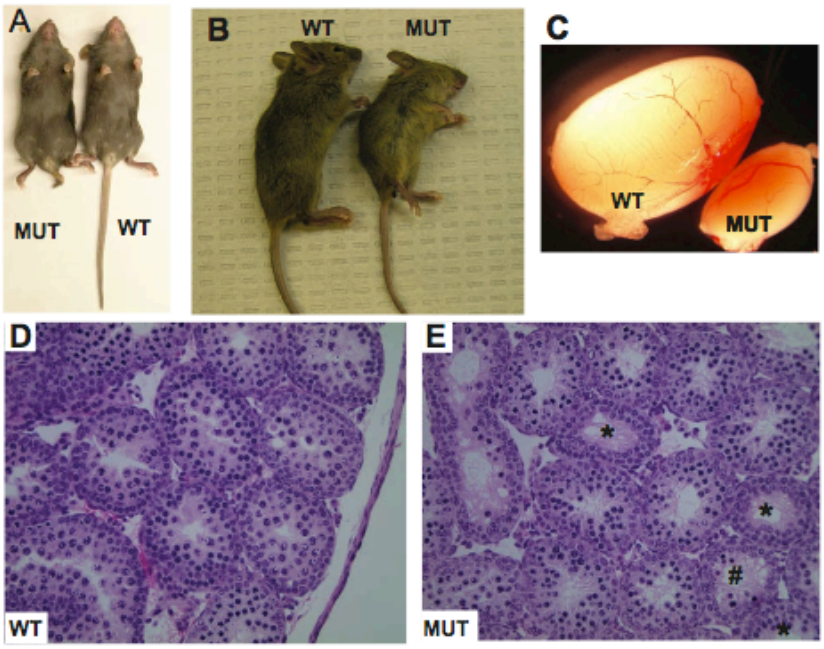



**Figure 2.** Developmental phenotypes of *Trip13* mutant mice.

(A) Shown are 21 day old littermates. Note the shortened tail in the mutant, but overall similar body size.

(B) Shown are 23 day old littermates. The mutant is smaller in this case, but the tail is not as truncated as the mouse in (A).

(C) Wild-type (WT) and homozygous *Trip13* mutant (MUT) testes.

(D) and (E) are cross sections through 17.5 day old heterozygous (”WT”) and homozygous mutant Trip13 testes, respectively. Whereas the tubules in WT show coordinated spermatogenesis with pachytene spermatocytes present in all tubules (proximal to the lumen), developmental progression in the mutant is not synchronized between tubules. Some tubules have no pachytene spermatocyes (asterisks), while others development is somewhat disorganized (#).

**Infertility due to meiotic disruption in TRIP13-deficient meiocytes**

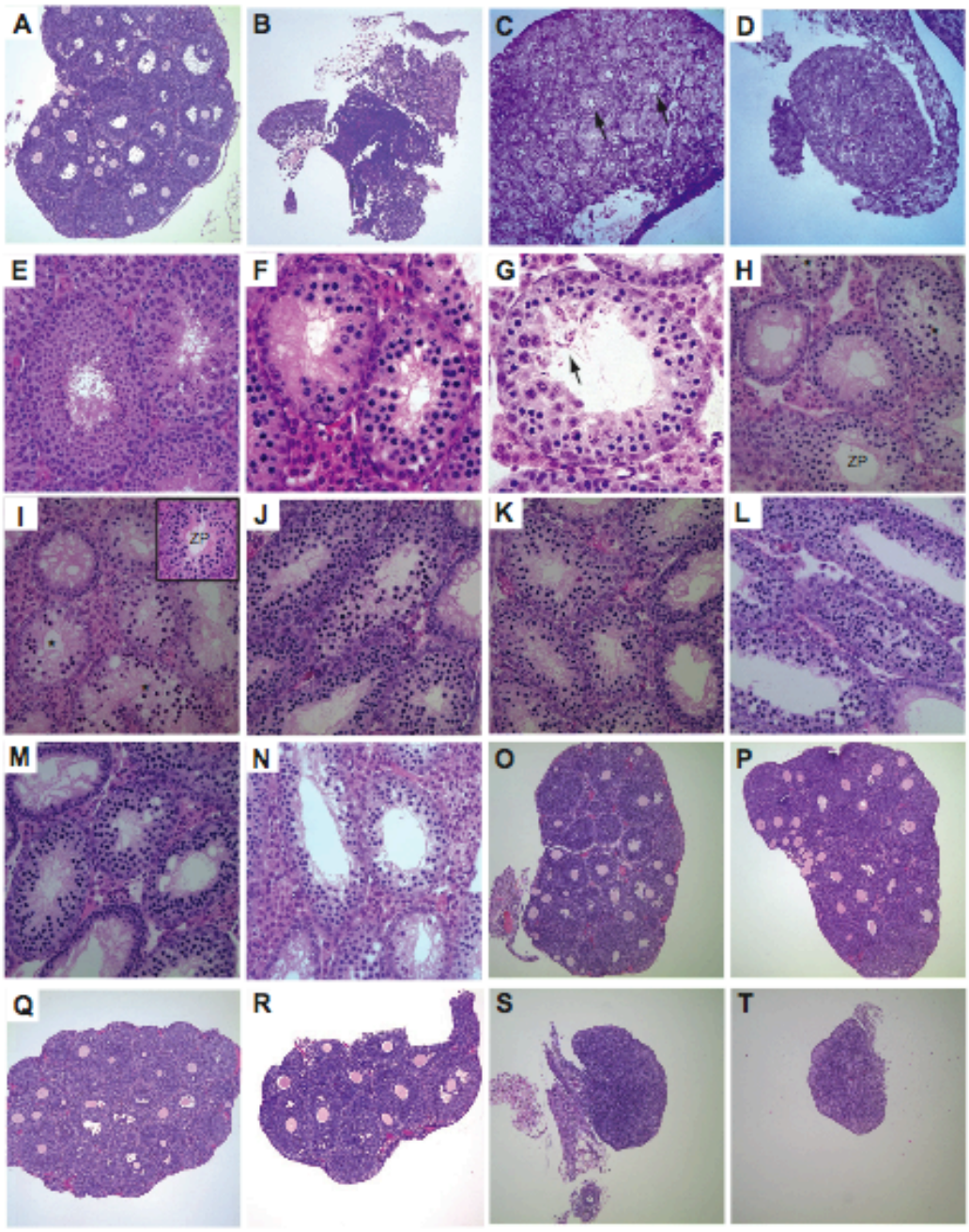
Homozygotes of both sexes had small gonads (Figure 2C; see below) and were invariably sterile. Ovaries of adult *Trip13*$^{Gt/Gt}$ females were severely dysmorphic and had few or no follicles (Figure 3A,B). The majority of oocyte loss occurred in late embryogenesis or early in postnatal development, since 2 day postpartum (dpp) ovaries were markedly smaller than those of control littermates, and were lacking oocytes or newly forming follicles (Figure 3C,D). Thus, oocytes failed to progress to the dictyate (resting) phase. Since I observed oocytes with pachytene stage chromosomes in 17.5 day *Trip13*$^{Gt/Gt}$ embryonic ovaries (not shown), this indicates that oocytes were eliminated somewhere between pachynema and dictyate.

Histological sections of mutant testes revealed a lack of postmeiotic cell types that are characteristic of wild-type seminiferous tubules (Figure 3E). The most developmentally-advanced seminiferous tubules contained adluminal spermatocytes with condensed chromatin characteristic of pachynema (Figure 3F). The absence of coordinated spermatogenic progression beyond this stage is indicative of a pachytene arrest. This was revealed more clearly by chromosome analysis (see below). Some sections of adult seminiferous tubules contained postmeiotic spermatids (Figure 3G), although I saw no motile epididymal sperm. These drastic meiotic defects stand in contrast to yeast and *C. elegans*, where deletion of *Pch2* alone has minor effects on spore/gamete development [2,8].

**Figure 3.** Histology of mutant gonads.

All are hematoxylin/eosin-stained paraffin sections. Testes are from 6 week old males, except as indicated below.

(**A**) Wild-type 25 day old ovary. (**B**) *Trip13*$^{Gt/Gt}$ 25 day old ovary, showing dysgenesis from an absence of oocytes. (**C**) 2 day old *Trip13*$^{Gt}$/+ control ovary. Arrows point to oocytes in newly forming follicles. (**D**) 2 day old *Trip13*$^{Gt/Gt}$ ovary, dysgenic due to lack of oocytes. Magnification is the same as its littermate in "**C**". (**E**) Wild-type testis. (**F**) *Trip13*$^{Gt/Gt}$ testis with uniform pachytene arrest. (**G**) *Trip13*$^{Gt/Gt}$ 3 month old testis with some postmeiotic spermatids (arrows). (**H**) *Spo11*$^{-/-}$ testis. A tubule with spermatocytes at leptotene/zygotene transition is labeled ZP, and tubules with apoptotic spermatocytes are marked with an asterisk. The specimen was taken from a littermate of that in (**I**). (**I**) *Spo11*$^{-/-}$ *Trip13*$^{Gt/Gt}$ testis. Labeling is the same as in "H". The inset contains a tubule with leptotene-zygotene spermatocytes. (**J**) *Mei1*$^{-/-}$ *Trip13*$^{Gt}$/+ testis. The specimen was taken from a littermate of that in (**K**). (**K**) *Mei1*$^{-/-}$ *Trip13*$^{Gt/Gt}$ testis. (**L**) *Rec8*$^{Mei8}$/*Rec8*$^{Mei8}$ *Trip13*$^{Gt}$/+ testis. The *Rec8*$^{Mei8}$ allele has been described [39]. The specimen was taken from a littermate of that in (**M**). (**M**) *Rec8*$^{Mei8}$/*Rec8*$^{Mei8}$ *Trip13*$^{Gt/Gt}$ testis. (**N**) *Dmc1*$^{-/-}$ *Trip13*$^{Gt/Gt}$ testis. (**O**) *Spo11*$^{-/-}$ *Trip13*$^{Gt}$/+ 25 day old ovary. The specimen was taken from a littermate of that in (**P**). (**P**) *Spo11*$^{-/-}$ *Trip13*$^{Gt/Gt}$ 25 day old ovary. (**Q**) *Mei1*$^{-/-}$ *Trip13*$^{Gt}$/+ 25 day old ovary. The specimen was taken from a littermate of that in (**R**). (**R**) *Mei1*$^{-/-}$ *Trip13*$^{Gt/Gt}$ 25 day old ovary. (**S**) *Rec8*$^{Mei8}$/*Rec8*$^{Mei8}$ *Trip13*$^{Gt}$/+ 25 day old ovary. The specimen was taken from a littermate of that in (**T**). (**T**) *Rec8*$^{Mei8}$/*Rec8*$^{Mei8}$ *Trip13*$^{Gt/Gt}$ 25 day old ovary.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**TRIP13-deficient meiocytes undergo homologous chromosome synapsis despite the presence of unrepaired DSBs in pachynema**

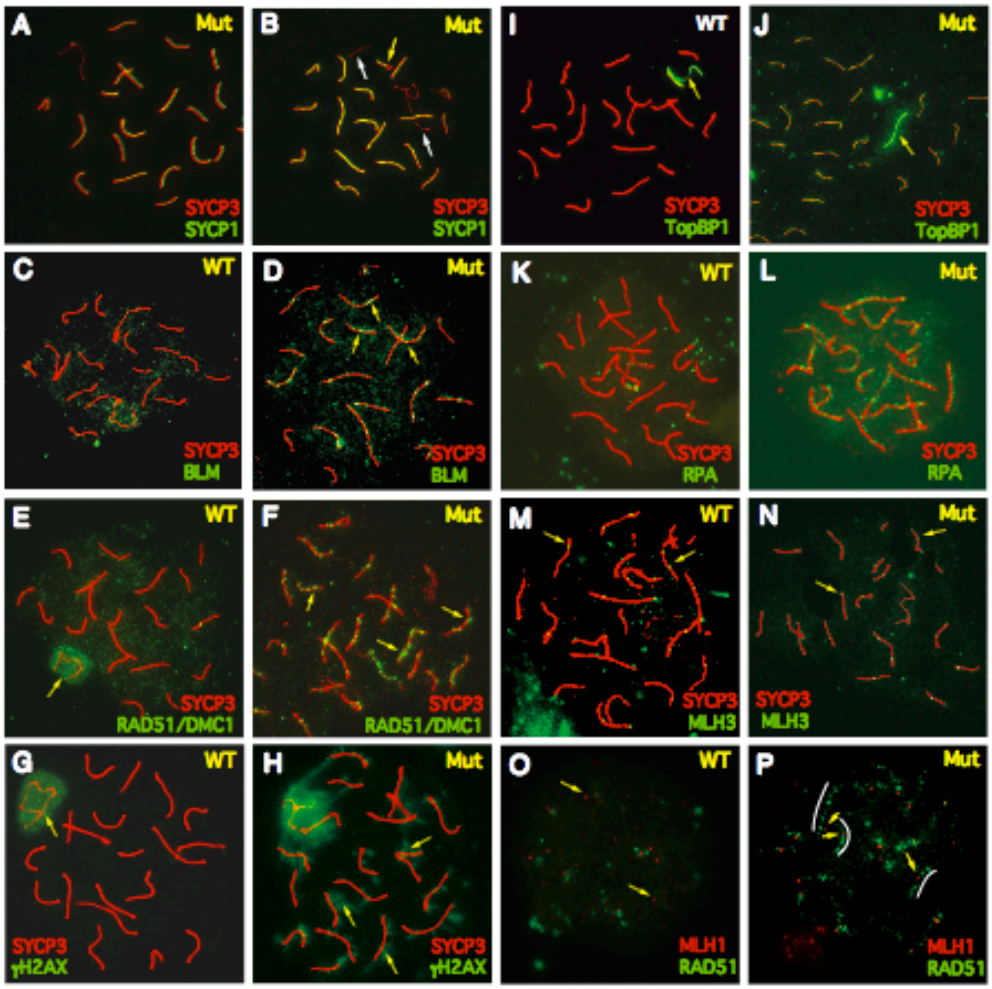
To better characterize the degree of meiotic progression in *Trip13*$^{Gt/Gt}$ spermatocytes, I immunostained chromosome spreads for SYCP3 and SYCP1, components of the axial/lateral elements and transverse filaments, respectively, of the synaptonemal complex (SC). Pachytene spermatocyte nuclei from postpubertal mutant testes could assemble normal SC cores and exhibited full synapsis of chromosomes as judged by co-labeling of SYCP1 and 3 along the full lengths of all autosomes (Figure 4A). Additionally, the X and Y chromosomes were normally synapsed at their pseudoautosomal region. More prepubertal (17.5 dpp) mutant spermatocytes contained asynaptic or terminally asynapsed chromosomes than age-matched controls (62.5% vs. 25%, respectively; Figure 4B). I attribute this to a delay in the first wave of postnatal spermatogenesis (Figure 2D,E), likely related to systemic developmental retardation (Figure 2A,B). Nevertheless, since *Trip13*$^{Gt/Gt}$ spermatocytes progress to pachynema with no gross SC abnormalities, and oocytes were eliminated soon after birth (a characteristic of DNA repair mutants [13]), this suggested that unrepaired DSBs are responsible for eventual meiotic arrest and elimination.

To elucidate the cause of meiotic arrest, I analyzed meiotic chromosomes with a variety of markers that are diagnostic of recombination and synapsis. Recombination in *Trip13*$^{Gt/Gt}$ spermatocytes appeared to initiate normally as judged by the presence of γH2AX in leptonema (Figure S2A,B) that reflects the presence of meiotically-induced DSBs [18]. RAD51 and/or DMC1, components of early recombination nodules (ERNs), was also present as abundant foci in *Trip13*$^{Gt/Gt}$ zygotene spermatocytes (not shown; the anti-RAD51 antibody cross-reacts with DMC1), indicating that recombinational repair of DSBs is initiated. The cohesin complex, which is essential

for completion and/or maintenance of synaptic associations, appeared to assemble normally as judged by immunolabeling for the meiosis-specific cohesins STAG3 (Figure S2C,D) and REC8 (not shown). Because yeast PCH2 localizes to telomeres in a Sir3p-dependent manner, I tested for possible telomere defects by immunolabeling for TRF2, a component of a protein complex that plays an essential role in telomere protection [19]. It was localized to telomeres of both fully synapsed and telomerically asynaptic mutant chromosomes (Figure S2E,F).

Defects in DSB repair became apparent in pachynema, upon probing mutant spermatocyte nuclei with antibodies against molecules involved in various stages of recombination. In >99% of *Trip13*$^{Gt/Gt}$ of chromosome spreads, BLM helicase (Figure 4C,D), RAD51/DMC1 (Figure 4E,F), γH2AX (Figure 4G,H), and TOPBP1 (Figure 4I,J) all persisted abnormally on synapsed chromosomes. For RAD51/DMC1, mutant pachytene spermatocytes contained 138 ± 6 foci (compared to 11 ± 3 in wild-type, most of which were on the XY body), down from 218 ± 13 in zygonema (compared to 220 ± 13 in wild-type). TOPBP1 is a DNA damage checkpoint protein involved in ATM-dependent activation of ATR [20,21]. It binds sites of DSBs and unsynapsed regions of meiotic chromosomes [22,23]. BLM has been reported to co-localize with markers (RPA and MSH4) of recombination at sites distinct from those that become resolved as crossovers (CO) [24]. I therefore assessed the distribution of RPA, the ssDNA binding protein, which is normally present at focal sites of synapsing meiotic chromosomes before disappearing in mid-pachynema [25]. It is thought to bind D-loops of recombination intermediates [26]. RPA also persisted on pachytene mutant chromosomes (Figure 4K,L). These data indicate that unrepaired DSBs, or unresolved recombination intermediates, remain in pachynema and activate a DNA damage checkpoint system.

**Figure 4.** Immunohistochemical analysis of pachytene spermatocyte chromosomes. Surface-spread chromosomes were immunolabelled with the indicated antibodies and fluors. As indicated in the upper right of each panel, cells were from wild-type (WT; either +/+ or *Trip13*$^{Gt/+}$) or *Trip13*$^{Gt/Gt}$ ("Mut"). There were no differences seen between heterozygotes and +/+ spermatocytes. (**A**) A mutant pachytene nucleus with full synapsis. Areas of SYCP1/SYCP3 co-labelling are yellow. (**B-E**) Spermatocytes nucleus from 17.5 dpp mutant. Asynapsed chromosomes or regions of chromosomes are indicated by white and yellow arrows, respectively. Unlike the normal distribution in wild-type pachytene spermatocytes (**C**), BLM foci are present on synapsed pachytene chromosomes in the mutant (**D**). RAD51 foci, which are abundant earlier in prophase, disappear from autosomes in wild-type pachytene nuclei (**E**) and the bulk of staining is over the XY-body (arrow). (**F**) RAD51 persists on the synapsed mutant chromosomes (arrows). (**G**) H2AX phosphorylation is restricted to the XY body in WT. (**H**) In addition to a large area of γH2AX staining (arrow) over the XY body, there is extensive autosomal H2AX phosphorylation (arrows). (**I, J**) Note that in wild-type pachytene spermatocytes, TOPBP1 is present only over the XY body (yellow arrow). In the mutant (J), an arrow denotes one area of intensive staining that may be over the sex chromosomes, but many other chromosome cores are positively stained. (**K, L**) RPA persists along synapsed cores in the mutant, not WT. (**M, N**) Arrows indicate examples of MLH3 foci on SCs. (**O**) In WT late pachytene spermatocytes, RAD51 is present only at background levels. (**P**) As in panel **F**, extensive RAD51 staining delineates SCs in mutant pachytene nuclei (indicates by white arcs). MLH1 foci co-localize with these tracts (arrows) at the typical 1-2 foci per chromosome as in **M**.


A
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SYCP3
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SYCP3
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RPA
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SYCP3
RPA
E
WT
SYCP3
RAD51/DMC1
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RAD51/DMC1
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SYCP3
MLH3
N
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SYCP3
MLH3
G
WT
SYCP3
γH2AX
H
Mut
SYCP3
γH2AX
O
WT
MLH1
RAD51
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RAD51

It should be noted that chromosomes affected by meiotic sex chromosome inactivation (MSCI) and meiotic silencing of unpaired chromatin (MSUC) are heavily stained by antibodies for several DSB repair-associated molecules, including γH2AX. H2AX phosphorylation due to MSCI and MSUC is conducted by ATR, not ATM [27-29]. Since mutant chromosomes are fully synapsed, and MSUC is known to occur only as a result of asynapsis, the decoration of *Trip13*$^{Gt/Gt}$ chromosomes with DNA repair markers is probably attributable to incomplete DNA repair rather than transcriptional silencing.

Consistent with the presence of rare (<1%) *Trip13*$^{Gt/Gt}$ pachytene spermatocytes devoid of persistent DNA repair markers, and testis histology showing some degree of postmeiotic progression (Figure 3G), I observed both diplotene nuclei that lacked autosomal RAD51/DMC1 and γH2AX (Figure S3A-D), and also metaphase I spreads with 20 bivalents (Figure S3E,F). Since *Trip13*$^{Gt}$ may not be a complete null, these diplotene and metaphase I spermatocytes might arise by virtue of having sufficient wild-type TRIP13.

**Crossover-associated markers appear normally in the absence of TRIP13**

The persistence of BLM on *Trip13*$^{Gt/Gt}$ spermatocyte chromosomes suggests that at least a subset of the unrepaired DSBs correspond to sites of defective noncrossover recombinational (NCO) repair. To assess whether CO recombination occurs in the mutant, I examined the distribution of the mismatch repair proteins MLH1 and MLH3, which are normally detectable as foci in mid-late pachynema and mark the locations of chiasmata [30,31]. Remarkably, MLH1/3 foci were formed; I observed 1-2 foci/chromosome as in wild-type and at typical overall levels (MLH3 = 23 ± 2, N=10; [30,32]) on mid-late pachytene chromosomes (Figure 4M,N; MLH1 not shown). Since <1% of *Trip13*$^{Gt/Gt}$ pachytene nuclei had normal repair (as judged by absence of

persistent DSB repair markers; see above), but most of the pachytene nuclei had MLH1/3 foci, it was unlikely that the MLH1/3 foci formed only on chromosomes with fully repaired DSBs. To test this directly, I conducted double staining for MLH1 and RAD51/DMC1. MLH1 foci were present on chromosomes that also contained numerous RAD51/DMC1 foci (Figure 4O,P).

To assess whether these MLH1/3 foci in *Trip13*$^{Gt/Gt}$ pachytene spermatocytes represent crossover events completed to a point where they could maintain interhomolog attachments though the end of prophase I, I treated testicular cells from 17.5-20.5 day old control (+/+), *Trip13*$^{Gt/Gt}$ and *Dmc1*$^{-/-}$ mice with the protein phosphatase inhibitor okadaic acid, a chemical that induces degradation of the SC, chromosome condensation, and premature progression to metaphase I [33]. Fifteen metaphase spreads were identified for each genotype. Whereas all of the *Dmc1*$^{-/-}$ spreads had ~35 or more condensed chromosomes, all of the +/+ and *Trip13*$^{Gt/Gt}$ spreads had 20-25, suggesting that the MLH1/3 foci in *Trip13*$^{Gt/Gt}$ pachytene spermatocytes represent sites of completed, or near-completed, crossovers. Because the preparations were made from whole testes, it is possible that the univalent-containing metaphases from *Dmc1*$^{-/-}$ mice were from spermatogonia, not spermatocytes.

**TRIP13 deficiency does not alleviate meiotic arrest phenotypes of mutants defective in synapsis.**

To determine if TRIP13 deficiency prevents apoptosis triggered by asynapsis as in *C. elegans*, I analyzed mice that were doubly mutant for *Spo11* and *Trip13*. SPO11 is a transesterase that is essential for the creation of genetically programmed DSB during leptonema of many organisms, including mice [18]. In *C. elegans*, *spo-11* mutant gametes have extensive asynapsis, which triggers PCH-2 dependent apoptosis in

pachynema [2]. In mice, *Spo11*$^{-/-}$ spermatocytes are severely defective in homologous chromosome synapsis [34,35], and arrest with chromosomes in a state characteristic of the zygotene/pachytene transition (Figure 3H). Spermatocytes in *Trip13*$^{Gt/Gt}$ *Spo11*$^{-/-}$ testes progressed maximally to that point before undergoing death (Figs 3I), well before the spindle checkpoint that eliminates achiasmate spermatocytes [36]. There was no evidence of metaphase I spermatocytes or postmeiotic spermatids in these testes, unlike those seen in *Trip13* single mutants (Figure 3G). In contrast to the complete synapsis in *Trip13*$^{Gt/Gt}$ pachytene spermatocytes (Figure 5A), in which SPO11 is available in leptonema to initiate (via DSB induction, Figure S2A,B) a recombination-driven homolog search, chromosome synapsis in doubly mutant spermatocytes was highly disrupted as in *Spo11* single mutants (Figure 5B,C). Identical studies were performed with mice deficient for *Mei1*, a vertebrate-specific gene also required for DSB formation and chromosome synapsis [37], with similar results (Figure 3J,K; immunocytology not shown).

In yeast, deletion of PCH2 alleviates the pachytene arrest caused by asynaptic mutants *zip1* and *zip2* [8]. Although mouse SYCP1 might be a functional equivalent of Zip1p, because *Sycp1* mutant spermatocytes arrest at approximately the same point as *Trip13* mutants, there would be no opportunity to observe bypass of *Sycp1*$^{-/-}$. Since Zip2p is present at sites of axial associations, even in *zip1* mutants, it has been suggested that Zip2p promotes initiation of chromosome synapsis [38]. These observations raise the possibility that in yeast, Pch2p responds to synapsis polymerization rather than initiation. To test this, I performed epistasis analysis with a *Rec8* allele (*Rec8*$^{Mei8}$, abbreviated as *Rec8*$^{-}$). Meiotic chromosomes of *Rec8* mutant spermatocytes undergo apparent homolog pairing and interhomolog synaptic initiation, but are defective in DSB repair and fail to maintain interhomolog synapsis [39,40]. Rather, sister chromatids appear to synapse and are bound by SYCP1 along

their axes. *Rec8* mutants do not progress to diplonema or metaphase I. Double mutant analysis indicated that *Rec8* is epistatic to *Trip13*. As in the *Spo11* & *Mei1* experiments, histology of testes deficient for both REC8 and TRIP13 resembled the *Rec8* mutant, with no evidence of progression to MI that occurs in $Trip13^{Gt/Gt}$ mice (Figure 3L,M). Immunocytological analysis of spread chromosomes showed a failure of homologous chromosome synapsis in both the $Rec8^{-/-}$ and $Rec8^{-/-}$ $Trip13^{Gt/Gt}$ spermatocytes, as previously reported for *Rec8* mutants (Figure 5D,E)[39,40].

Although subsequent reports indicate otherwise [10,12], deletion of *PCH2* in yeast was originally reported to alleviate meiotic arrest caused by deficiency for the meiosis-specific RecA homolog *DMC1* [8]. To investigate this relationship in mice, I constructed animals doubly mutant for *Trip13* and *Dmc1*. As in $Dmc1^{-/-}$ mice, where spermatocytes undergo meiotic arrest from defective DSB repair and failed chromosome synapsis [16], spermatogenesis in $Dmc1^{-/-}$ $Trip13^{Gt/Gt}$ testes was uniformly arrested at the point where spermatocytes contained chromatin characteristic of zygonema/pachynema (Figure 3N). Immunocytological analysis indicated that both $Dmc1^{-/-}$ and $Dmc1^{-/-}$ $Trip13^{Gt/Gt}$ chromosomes had extensive asynapsis compared to $Trip13^{Gt}$ single mutants (Figure 5f-h), and all had persistent RAD51/DMC1 foci and phosphorylated H2AX (γH2AX; Figure 5I-L), confirming that *Dmc1* is epistatic to *Trip13*. Doubly mutant females had residual ovaries, phenocopying $Dmc1^{-/-}$ and $Trip13^{Gt/Gt}$ single mutants (not shown).

**Meiotic defects in $Trip13^{Gt/Gt}$ oocytes are DSB-dependent**

Epistasis analysis of females was insightful with respect to the cause of arrest in *Trip13* mutants. Both $Mei1^{-/-}/Trip13^{Gt/Gt}$ and $Spo11^{-/-}/Trip13^{Gt/Gt}$ females had ovaries with numerous follicles, identical to *Mei1* and *Spo11* single mutants (Figure 3O-R). Thus, *Spo11* and *Mei1* are epistatic to *Trip13* in oogenesis, just as they are to *Dmc1*

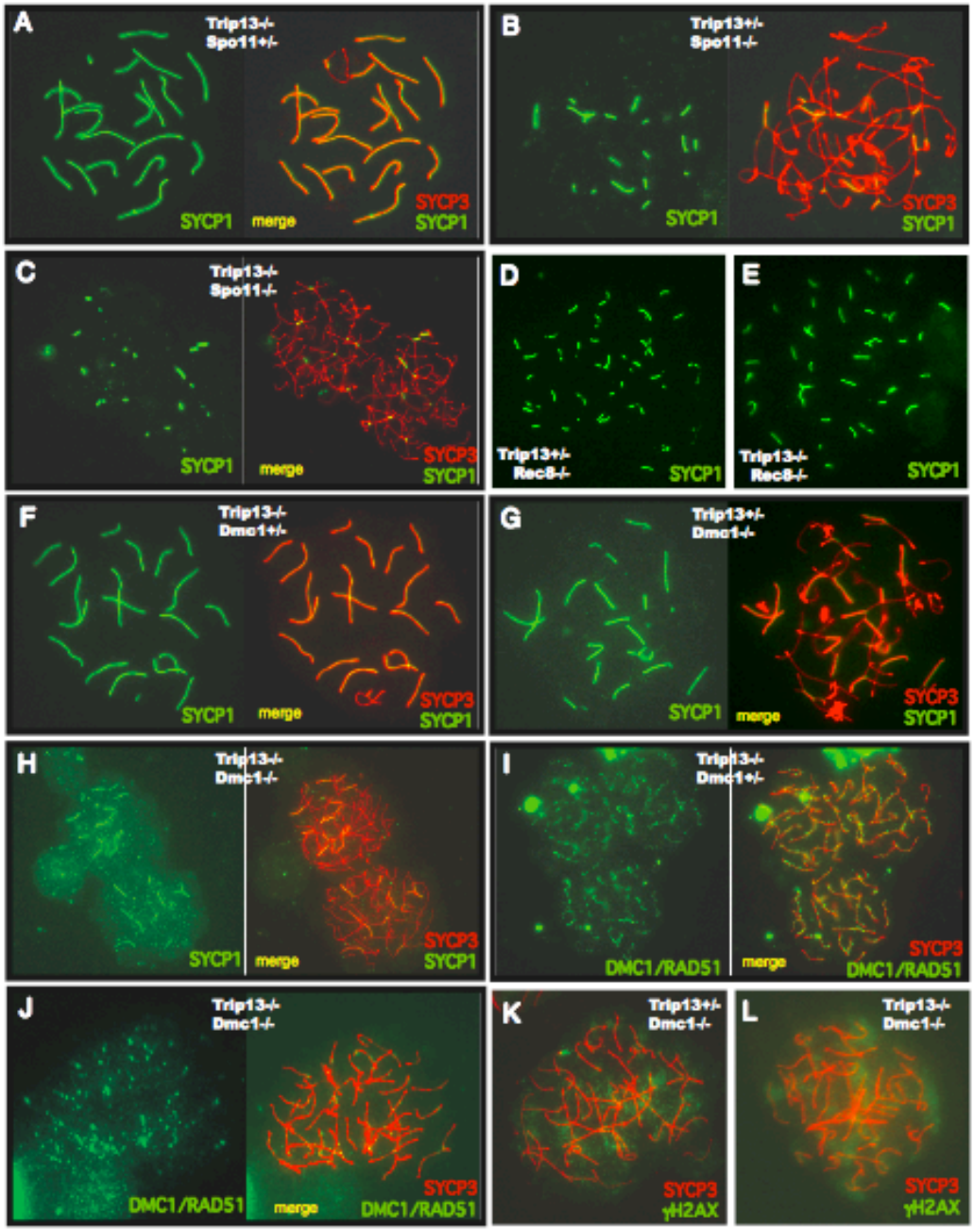


**Figure 5.** Immunocytological analysis of *Trip13* compound mutants. Surface-spread chromosomes were immunolabelled with the indicated antibodies and fluors. Genotypes are indicated, as are those panels in which dual staining patterns are merged. Note that panels H & I are at lower magnification to show multiple nuclei.

[13,41]. This demonstrates that oocyte loss in *Trip13*$^{Gt/Gt}$ females is dependent on DSB formation. In conjunction with the immunohistochemical data, these data provide strong evidence that meiotic arrest in *Trip13* mutant mice is due to defects in DSB repair. As expected, ovaries of *Rec8 Trip13* double mutants were devoid of oocytes as were those from either single mutant (Figure 3B,S,T).

## DISCUSSION

Genetic experiments in *S. cerevisiae* provided evidence that the pachytene checkpoint monitors and responds to recombinational DSB repair and synapsis independently. Wu and Burgess concluded that the repair checkpoint is *RAD17*-*SAE2*-dependent, while the synapsis checkpoint is *PCH2*-*ZIP1*-dependent [12]. Of these 4 genes, *SAE2* and *ZIP1* do not have clear mammalian orthologs (although SYCP1 may be a functional ortholog of ZIP1), and mutation of the mouse *RAD17* ortholog, *Rad1*, presumably causes embryonic lethality [42]. Thus, mutational analysis of mouse *Pch2* (*Trip13*), which is also critical for the synapsis checkpoint in *C. elegans* [2], was the best remaining option to evaluate potential functional conservation in mammalian meiotic checkpoint control.

Our results demonstrate that in mice, the primary meiotic function of TRIP13 is in recombination itself. I found no evidence that it is involved in pachytene checkpoint control. Our data suggests that while recombination events destined to be resolved as crossovers can proceed normally in *Trip13* mutants, DSBs that enter the NCO repair pathway are incompletely resolved or processed inefficiently. This hypothesis is compatible with current knowledge of meiotic recombination pathways. In *S. cerevisiae*, CO and NCO pathways are distinct [43]; they have different recombination intermediates, and are dependent upon different proteins [44,45]. Mice also appear to

have independent CO vs. NCO recombination pathways [46]. As in yeast, both require SPO11-induced breaks, but only the CO pathway requires MLH1. Both types of recombinant products are formed by mid-late pachynema. Another possibility is that the recombination defects are a result of defective intersister recombination. However, this type of DSB repair is suppressed in meiotic cells. Ablation of *RAD54*, which mediates intersister recombination in yeast, does not significantly disrupt meiosis in either yeast or mice [47,48]. Interestingly, RAD54-deficient spermatocytes display abnormal persistence of RAD51 foci on pachytene chromosomes, similar to those in TRIP13 mice, but there are no deleterious effects on meiotic progression or fertility [49].

Data from budding yeast also indicate that Pch2p functions in recombination. Deletion of *PCH2* delays meiotic progression by ~2 hours in SK1 yeast, and causes a minor decrease in ascus formation [50]. DSBs persist >2 hours longer in *pch2Δ* yeast than in wild-type, and hyperresection of DSBs in *dmc1Δ* cells is decreased in *dmc1Δ pch2Δ* double mutants [10]. Additionally, it was reported that *pch2Δ* yeast had a meiosis I delay dependent on the *RAD17-SAE2* checkpoint that monitors recombination intermediates [12]. However, the exact role of TRIP13 (or Pch2) in recombination is unclear. Because synapsis occurs in TRIP13-deficient spermatocytes and is dependent on DSB formation (activity of SPO11 and MEI1), I suggest that TRIP13 functions after homology recognition and strand exchange, and that recombination events entering the CO repair pathway are either completed or nearly so (because okadaic acid treated resulted in bivalent chromosomes). One possibility for TRIP13's role in recombination is that it is directly involved in a step specific to resolution of NCO recombination intermediates. Another possibility is that TRIP13 is required for disassembly of NCO recombinational repair complexes [51] containing those proteins that persist abnormally on *Trip13*$^{Gt/Gt}$ pachytene chromosomes.

Notably, TRIP13 has two putative ATPase domains, a signature of AAA-ATPase ClpA/B chaperones that perform protein or protein/DNA complex disassembly [52]. These potential recombination roles might not be limited to meiosis, since *Trip13* is widely transcribed and the mutant animals exhibited developmental defects. Finally, TRIP13 might play an indirect role, such as providing a "licensing" signal for the resolution of NCO intermediates and completion of meiosis.

Regarding the cause of cell death in *Trip13* mutants, our data indicate that this is triggered by defective DSB repair rather than asynapsis. I base this conclusion on two observations: 1) oocyte elimination is dependent upon DSB formation, and 2) synapsis is normal in spermatocytes of adult testes. Indeed, this mutant is unique in that recombination defects occur in the absence of asynapsis (*e.g.* as in *Dmc1* knockouts). Thus, the *Trip13* mutant provides the first evidence that unrepaired DNA damage alone can trigger the mammalian pachytene checkpoint response. Furthermore, our results allow us to conclude that oocytes and spermatocytes share a similar, if not identical, DNA damage pachytene checkpoint that is decoupled from a synapsis checkpoint.

Interestingly, I found that okadaic acid (OA) treatment of *Trip13*$^{Gt/Gt}$ spermatocytes could propel them into MI, despite a report that the same did not occur when wild-type pachytene spermatocytes were treated with the DSB-inducing agents gamma radiation or etoposide [53]. It is possible that the nascent induction of DSBs in pachynema evokes a checkpoint response that cannot be bypassed by OA, whereas the post-strand-invasion lesions in TRIP13-deficient spermatocytes do not.

TRIP13 was originally discovered as an interactor with rat thyroid receptor beta (THRb; [54]), but the relationship between THRb and TRIP13 in meiosis is unknown. Interestingly, I observed that THRb is distributed diffusely throughout wild-type spermatocyte nuclei but is excluded from the XY (sex) body (unpublished

observations), a compartmentalized nuclear domain beginning in pachynema, in which the sex chromosomes become heterochromatinized and transcriptionally silenced in a process called meiotic sex chromosome inactivation (MSCI; [55]). However, the XY body appeared intact in most mutant spermatocytes upon probing with several markers of XY heterochromatinization (unpublished observations). Considering that THRb knockout mice are viable and fertile [56], the functional relationship between TRIP13 and its receptor THRb in meiosis is unclear.

Given the high similarity of PCH2 orthologs throughout the eukaryotic world, one or more essential functions of this protein must be conserved. Since TRIP13 does not exhibit checkpoint function in mice, I surmise that the TRIP13/PCH2 ancestral protein had a function in recombination that persists to the present. Notably, *Arabidopsis thaliana* does not appear to have a meiotic checkpoint activity that eliminates mutant meiocytes in a manner analogous to organisms such as mice, budding yeast and female *Drosophila* [11,57], and mammalian TRIP13 is more similar to *Arabidopsis* PCH2 than the fly or worm proteins (Figs. 1A, S1). The unusual relatedness between mammalian and plant PCH2 may therefore be attributable to both the presence of a common conserved function (namely recombination, though the role of PCH2 in plants has yet to be determined), and the absence of checkpoint function. Nevertheless, the evolutionary relationships between animals, fungi and plants (which are discordant with PCH2 sequence phylogeny) do not allow parsimonious models addressing the points in time that checkpoint functions in PCH2 were gained or lost. It is possible that its checkpoint function evolved independently in worms and budding yeast. The picture will become clearer as the function of PCH2 in other organisms is elucidated.

The nature of the synapsis checkpoint in male mice remains unidentified. One possible candidate is *Dot1* (a.k.a. *PCH1* in yeast), a histone methyltransferase

silencing factor that is required for pachytene arrest of *zip1* and *dmc1* mutants in yeast [58], and for preventing RAD54-mediated recombinational DSB repair between sister chromatids. However, *DOT1* acts upstream of *PCH2*. Given that TRIP13 doesn't have checkpoint function in mice, a potential role for mammalian DOT1 in the pachytene checkpoint is dubious but awaits investigation. Recently, it was shown that the TRP53 homolog TRP63 is required for DNA damage-induced death of dictyate-stage primordial oocytes, leading to the suggestion that it is involved in monitoring genome integrity [59]. However, this activity occurs subsequent to a pachytene checkpoint. As alluded to earlier, a complicating problem for studying potential meiotic checkpoint genes in mice is that as in yeast, such genes often have mitotic functions (such as RAD24 [7]), and their ablation can cause lethality [42]. Unless mammalian pachytene checkpoint components have orthologs with similar functions in organisms such as yeast, their identities are likely to remain elusive.

## MATERIALS and METHODS

### PCR analysis of *Trip13* cDNA

*Trip13* was amplified from samples of Clontech's Mouse Multiple Tissue cDNA Panel I, using the following primers: 5'-GCACCATTGCACTTCACATC-3' (TRP3-6F) and 5'-TGACCATCAGACTGTCGAGC-3' (TRP3-6R). These primers correspond to exons 3 and 6, respectively, and amplify a 330 bp cDNA product. The cDNAs in this panel are equalized to allow quantitative analysis by RT-PCR.

### Generation of *Trip13*-deficient Mice

The mouse embryonic stem (ES) cell line RRB047 (strain 129/Ola) containing a gene trap insertion in *Trip13* was obtained from BayGenomics

(Baygenomics.ucsf.edu/). The gene-trapping vector used to create this line, pGT1lxf, was designed to create an in-frame fusion between the 5' exons of the trapped gene and a reporter, *βgeo* (a fusion of β-galactosidase and neomycin phosphotransferase II). The gene-trapped locus creates a fusion transcript containing exons 1-3 of *Trip13* and *βgeo*. To identify the exact insertion site within intron 3, PCR reaction were performed using one primer within the gene trap vector, and the other primer at various positions in intron 3 pointing towards the 3' end of the gene. Product from a productive reaction was sequenced, revealing that the insertion site was 445 bp into intron 3.

**Genotyping of mice**

Three primers were used to distinguish wild-type and mutant alleles of *Trip13*:

Primer 1: CGTCGCTCCATTGCTTTGTGC

Primer 2: AGTAGTGGTACACTGTATTTTTGCTTTCATTGA

Primer 3: GTAGATCCCGGCGCTCTTACCAA

Primers 1 and 2 are located upstream and downstream, respectively, of the gene trap insertion within the intron 3. Primer 3 corresponds to pGTlxf sequence. Primers 1 and 2 amplify a 700bp band from a wildtype allele; primers 1 and 3 amplify a 540-bp fragment from a mutant allele. Separate reactions were used to assay the presence or absence of each amplicon from a DNA sample. The cycling conditions were: 94°C 2min; 94°C 30s, 57°C 45s, 72°C 50s for 35 cycle; 72°C 2 min.

**RT-PCR**

Total RNA was isolated from adult testis with the RNeasy Mini Kit (Qiagen, Valencia, CA), and 4.0μg was oligo dT primed and reverse-transcribed with Superscript II (Stratagene). The entire *Trip13* protein-coding sequence was amplified

with primers 5'-ATGGACGAGGCGGTG-3' and 5'-TCAAACATAAGCTGAAAGTT-3'. The cycling conditions were: 94°C 2min; 94°C 30s, 55°C 45s, 72°C 80s for 35 cycles, 72°C 2 min. The primers for amplifying the *Med31* coding sequence as control were : 5'-ATGGCCGCGGCCGTCGCTATGG-3' and 5'-TCATTTCCCTGCTGTGTTATTCTGCTGCTGCTGC-3'. The cycling conditions were: 94°C 2min; 94°C 30s, 55°C 30s, 72°C 35s for 35 cycles; 72°C 2 min.

**Development and purification of chicken antibodies**

A peptide corresponding to amino acids 25-40 of TRIP13, VLQRSGSTAKKEDIK, was conjugated to KLH, and used to immunize chickens (done by Sigma Genosys). Polyclonal IgY was isolated from eggs with the Eggcellent Chicken IgY Purification kit (Pierce). IgY antibodies were then affinity purified using the immunizing synthetic peptide.

**Western blotting**

50μg of testis extract in RIPA buffer was separated by 8% SDS-PAGE and electrotransferred onto a Pure Nitrocellulose membrane (Bio-Rad). The membrane was incubated with a polyclonal rabbit anti-human TRIP13 antibody (Genway; Cat# 18-003-42687). According to the manufacturer, the immunogen was a synthetic peptide embedded in sequence I deduced to correspond to exon 3. Binding was detected by chemiluminescence ECL kit (Pierce) using a rabbit anti-chicken IgG horseradish peroxidase conjugate (Pierce).

**Histological Analyses**

Testes or ovaries were fixed in Bouin's, embedded in paraffin, sectioned at 6 μm, and stained by hematoxylin and eosin. Antigen retrieval for immunohistochemistry of

testis sections was as described [60]. Oocyte and follicle numbers were counted as described [61]. Only follicles containing an oocyte with a clearly visible nucleus were scored.

**Immunocytochemistry**

Immunolabeling of surface-spread spermatocytes and oocytes was performed as described [39,62]. To reach conclusions on the pattern of staining for various proteins, 30 (unless otherwise indicated) well-spread nuclei of particular meiotic stages were first identified under the fluorescent microscope on the basis of SYCP3 or STAG3 staining, then imaged at both appropriate wavelengths to determine the pattern of second proteins with focal patterns such as RAD51 or RPA. Unless otherwise indicated, the panels shown in the figures were the exclusive or predominant patterns seen. The exception for this approach was in the case of staining for MLH1 or 3 plus RAD51 (in which case SYCP3 or STAG3 was not available to find chromosome cores). Nuclei in this situation were identified first by MLH1/3 foci clustering, then imaged for both fluorescent wavelengths.

Primary antibodies used in this study were as follows: mouse anti-SCP3 (1:500; Abcam); rabbit anti-SYCP1 (1:1,000; a gift from C. Heyting) [63]; rabbit anti-REC8 (1:100; a gift from C. Heyting); rabbit anti-RAD51 (1:250, this polyclonal antibody recognizes both RAD51 and DMC1, Oncogene Research Products); rabbit anti-γH2AX (1:500, Upstate Biotechnology); rabbit anti-STAG3 (1:1,000; a gift from R. Jessberger); rabbit anti-MLH3 (1:400; a gift from P. Cohen); mouse-anti-human MLH1 (1:50 BD Biosciences); rabbit-anti-TopBP1 (1:100, a gift from J. Chen) [22]; mouse-anti-ubiquityl-histone H2A (1:200; Upstate Biotechnology); rabbit-anti-TRF2 (1:500; a gift from Titia de Lange); and rabbit-anti-BLM (1:50, a gift from Raimundo Freire). All secondary antibodies conjugated with either Alexa Fluor 488 or 594

(Molecular Probes) were used at a dilution of 1:1000. All images were taken with a 100x objective lens under immersion oil.

**Metaphase I spermatocyte spreads and okadaic acid (OA) treatment.**

Metaphase fixed spermatocytes from 8 month old *Trip13*$^{RRB047}$ homozygotes, using 23 day old wildtype mice as control, were prepared and stained with Giemsa as described [64].

For OA treatment, cells were exposed to 5 μM OA (Calbiochem, San Diego, CA) for 6 h at 32 degrees in a humidified, environment of 5% $CO_2$ before spreading [65]. These preparations were stained with DAPI to visualize metaphase nuclei and chromosomes.

**Phylogenetic analyses**

TRIP13 orthologs were identified by BLASTP searches of Genbank and other sources providing gene models such as Ensembl. The selected orthologs can be found in Table S1. Amino acid alignments were done with Clustal W, using the default settings with and without removing the regions outside of the AAA-ATPase central domain. The trees were constructed by using the neighbor-joining method (NJ) with Poisson correction. The reliability of internal branches was assessed by using 500 bootstrap replicates, and sites with gaps were ignored in this analysis. NJ searches were conducted by using the computer program MEGA3 [66].

# APPENDIX

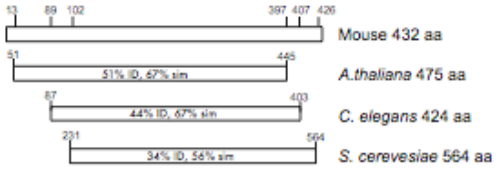


Figure S1. Depiction of conserved regions of mouse TRIP13 and its PCH2 orthologs.

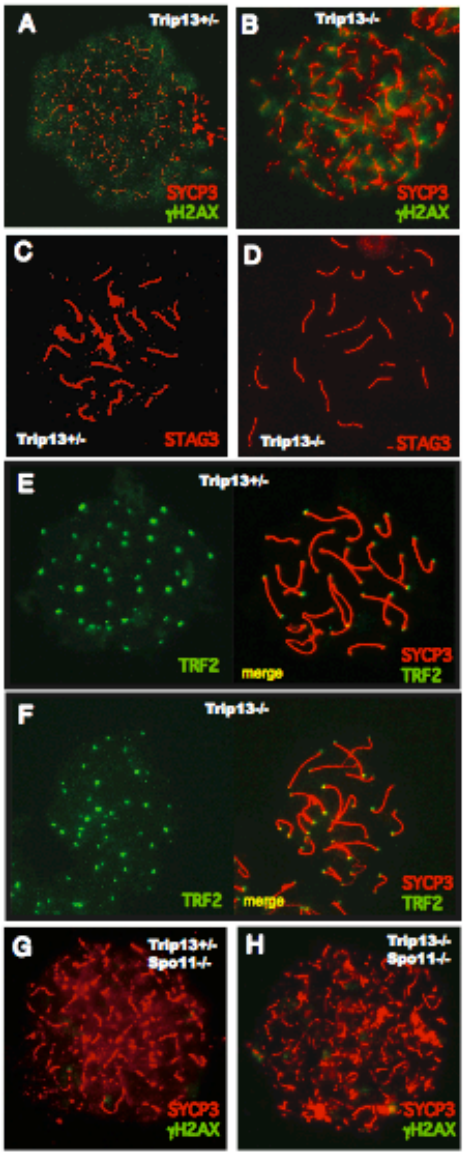


Figure S2. Surface-spread chromosomes were immunolabelled with the indicated antibodies and fluors.

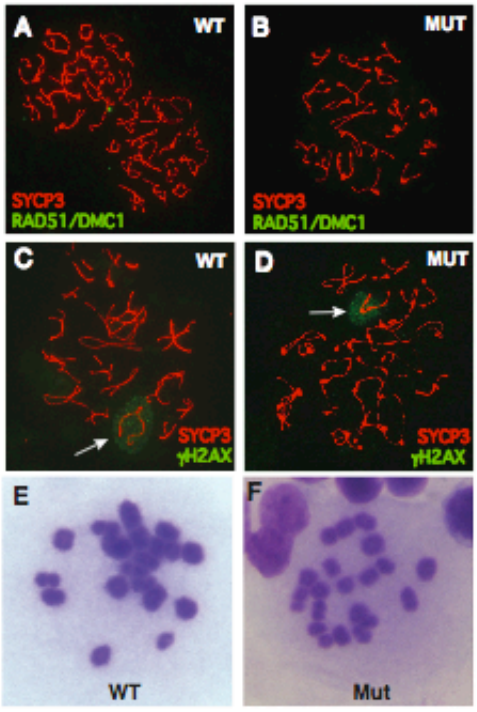


Figure S3. Trip13 mutant spermatocytes that progress beyond pachynema have repaired DSBs and form bivalents at Metaphase I.

Table S1. Sources of TRIP13 Amino Acid sequences used to construct the phylogenetic tree in Figure 1.

| Species | Protein/Gene ID | Gene Name |
|---|---|---|
| Bos taurus | ENSBTAG00000006972 | Trip13 |
| Caenorhabditis elegans | F10B5.5 | Pch2 |
| Canis familiaris | ENSCAFG00000010838 | 609426 |
| Ciona savignyi | ENSCSAVG00000007117 | novel Ensembl prediction |
| Danio rerio | ENSDARG00000025043 | Trip13 |
| Drosophila melanogaster | CG31453 | CG31453 |
| Gallus gallus | ENSGALG00000012508 | 420798 |
| Homo sapiens | ENSG00000071539 | Trip13 |
| Macaca mulatta | ENSMMUG00000014740 | Trip13 |
| Monodelphis domestica | ENSMODG00000000818 | Trip13 |
| Rattus norvegicus | ENSRNOG00000015810 | Trip13 |
| Saccharomyces cerevisiae | YBR186W | Pch2 |
| Takifugu rubripes | NEWSINFRUG00000136965 | Novel Ensembl prediction |
| Tetraodon nigroviridis | GSTENG00034091001 | GSTENG00034091001 |
| Xenopus tropicalis | ENSXETG00000013105 | Trip13 |
| Mus musculus | ENSMUSG00000021569 | Trip13 |
| Caenorhabditis briggsae | BP:CBG00732 | BP:CBP13887 |
| Anopheles gambiae | ENSANGT00000011549.3 | Q7PXQ3_ANOGA |
| Drosophila pseudoobscura | GA16260-PA | GA16260-PA |
| Oryza sativa | CAE05878 gi:38605777 | OSJNBa0044K18.20 |
| Arabidopsis thaliana | NP_194202 | AT4G24710.1 |
| Ashbya gossypii | NP_984603 | AEL258W |
| Debaryomyces hansenii | XP_460723 | DEHA0F09229g |
| Candida glabrata | XP_449627 | CAGL0M06435g |
| Kluyveromyces lactis | XP_455025 | unnamed protein product |

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